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A STUDY OF VIRUS-HOST INTERACTIONS
IN THE GENUS RHIZOBIUM

by

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A thesis submitted for the degree of Doctor of Philosophy.

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Dedication

To my wife, Christina; my mother and father and all genetic precursors; Burns, Maclean, MacDiarmid and all those "krassivy, krassivy" who have had a formative effect on my spirit and psychology.

Summary

Various rhizobial strains were isolated which were sensitive to the virulent rhizobiophage, ØC, and eight sensitive strains were tested for the presence of a restriction system. No restriction systems were found and variations in efficiencies of plating were attributable to adsorption phenomena and the selection of host range mutants. The virulent phage ØC, was found to have a broad host range because it plated on various rhizobial species, but even in interspecific phage growth studies no evidence of restriction was found.

Several rhizobia were screened for bacteriocinogeny and lysogeny. No evidence was found for widespread bacteriocinogeny but one strain, G28, released a phage which plated on strain G18. The phage responsible (ØS28) was temperate in G28 and G18. UV inactivation experiments suggested that ØS28 had a genome size comparable with that of ØC.

The polylysogenic Su297/Su298 system was investigated. Su297 released a phage (Øi) which plated on Su298 with low efficiency and the plaques produced on Su298 were due to a novel phage heteroimmune with Øi. The novel phage (Ø8) was temperate in Su297, contrary to the findings of previous workers. On infection of Su298 by Øi another phage was released. This phage was Ø7 and it was also heteroimmune with Øi. The biogenesis of Ø7 and Ø8 was studied and from the data several criticisms were made about the interpretation of earlier data by other workers. A recombinational model for Ø7 and Ø8 biogenesis was presented to explain the observed host range of the various phages.

Although transfection attempts proved totally ineffective, transduction attempts using the Ø7-related phages appeared to work. Analysis of the putative transductants revealed considerable heterogeneity in phage sensitivity patterns. A model was presented to explain the formation of such putative transductants.

CHAPTER I

GENERAL INTRODUCTION

"Bacteriologists say the soil's teemin' mair thrang
wi' life than at ony ither time, yet wi' nocht to
show."

Hugh MacDiarmid

Although it has been known for many years that the root nodule bacteria play an important role in the supply of fixed nitrogen to leguminous crops, most aspects of Rhizobium biology have been inadequately studied. There have been many reports on various aspects of rhizobial ecology (e.g. see Quispel, 1974) but comparatively little is known about the biochemistry and molecular biology of this genus. The rhizobia are important for two major reasons. They are capable of fixing atmospheric nitrogen into a form which is readily usable by their plant host and they are capable of existing in a state of symbiosis with leguminous plants.

Because Rhizobium can fix nitrogen for plant crops it is agriculturally important. This agricultural importance has been largely underestimated in the British agricultural economy, until recent times, due to the relatively low price of oil on world markets. Because, in the past, oil was cheap then fossil fuel based nitrogenous fertilizers were correspondingly cheap and so fixed nitrogen could be added to large areas of agricultural land at relatively low cost. The sudden increase in the world price of oil, and its maintenance at high levels, meant that far more attention was focussed on the provision of alternative energy sources for the production of fertilizers. Also, more attention was focussed on the process of biological nitrogen fixation (see Hardy and Havelka, 1975; Evans and Barber, 1977). Agriculture in U.S.A. and the U.K. is a multi-billion dollar industry and so financing research into the biology of nitrogen fixation is a sound economic investment to which there are political overtones which cannot be discussed here. In this respect it is interesting that the intensity of financial backing for nitrogen fixation research has effectively mirrored oil prices (Hardy and Havelka,

1975; Evans and Barber, 1977).

Rhizobium is one genus in a select group of organisms which can enter into symbiotic relationships with eukaryotic cells. Other prokaryotes which are capable of such symbiotic relationships with eukaryotes include the rumen bacteria of cows and the blue-green algal symbionts of lichen fungi (Stanier, Dondoroff and Adelberg, 1968). Consequently the rhizobia are of profound biological importance because very little is known about the complex interactions which take place in any symbiosis. Therefore the Rhizobium/legume relationship may act as an important model system for symbiosis in general.

Although the rhizobia can fix nitrogen symbiotically they only do so in conjunction with distinct groups of plants. The flowering plants of the Leguminosae are found in temperate and tropical areas and include a wide variety of plants from the small clovers to trees such as Acacia. Between 80 and 90% of the Papillionaceae, a subdivision of the Leguminosae, can form nodules in conjunction with Rhizobium. Of the other subdivisions, only 25% of the Mimosaceae and very few of the Caesalpinaceae can enter into symbiosis with the root nodule bacteria. Within the Papillionaceae the commonest plants to form nodules in symbiosis with Rhizobium include the peas, beans and clovers, hence the agricultural importance of the rhizobia.

What are the characteristics of Rhizobium?

In any critical assessment of rhizobial research several points must be borne in mind. It is important to know whether or not any particular study was actually conducted on Rhizobium. This may seem a ridiculously obvious point, but the rhizobia grow so slowly compared with most other bacteria that they are rapidly overgrown by contaminants in prolonged culture. The rhizobia can be classified into two types dependent on their growth rates. The "fast growers" generally divide every 2-4 hours and include R. meliloti, R. trifolii, R. leguminosarum and R. phaseoli. Whereas the "slow growers" divide

every 3-10 hours and include R. lupini, R. japonicum and the "cowpea rhizobia". Consequently, even with the fast growers, contaminants such as Klebsiella, Bacillus or Pseudomonas can rapidly overgrow the rhizobia. Because of this problem great care must be taken, to avoid contamination, when working with Rhizobium. Unfortunately there is only one definitive test which establishes the identity of Rhizobium and this is the nodulation test (see Vincent, 1970). Therefore in assessing publications on rhizobial research the presence or absence of such tests is of crucial importance. In cases where rhizobia defective in nodulation are used they must fulfill all other rhizobial characteristics. Other rhizobial characteristics include the slow growth rate; a mucoid colony appearance; rhizobiophage sensitivity; absence of pigmentation and inability to grow on nutrient agar, in most cases. Because of such identification and purity problems almost all of the advances in the fields of nitrogen fixation research have come from studies on bacteria which have proved more amenable to biochemical and genetic studies. In particular rapid advances have been made in the study of the molecular biology of biological nitrogen fixation in the fast growing bacterium Klebsiella (see page 7).

What are the key areas of rhizobial research?

Because of the importance of Rhizobium in several biological areas the key aspects of rhizobial research are the biochemistry of nitrogen fixation in Rhizobium; the molecular biology of rhizobial nitrogen fixation and the factors which affect the development of the Rhizobium/legume symbiosis. Unfortunately in Rhizobium very little was known about any of these fields until comparatively recently when two major breakthroughs were achieved. These breakthroughs were the discovery of symbiotic nitrogen fixation in Rhizobium (1975) and the development of recombinational genetic mapping in Rhizobium in 1976. Both aspects will be discussed in detail below.

Asymbiotic nitrogen fixation

A) A digression into Klebsiella

1. The biochemistry of nitrogen fixation

The enzyme responsible for the conversion of nitrogen gas to ammonia, as depicted in reaction (1), is called nitrogenase.

Nitrogenase, which is only found in some blue

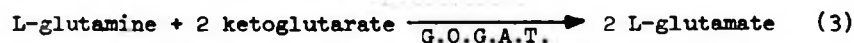
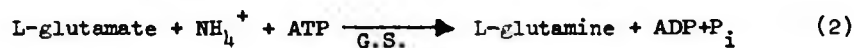


green algae and bacteria, has been isolated from a variety of nitrogen fixing organisms, and has been the subject of intensive investigation (for review see, Postgate, 1971; Dilworth, 1974; Burns and Hardy, 1975; Winter and Burris, 1976; Mortenson and Thorneley, 1979). The enzyme is composed of two proteins designated the "MoFe" and "Fe" proteins. The larger protein (MoFe) usually contains two atoms of molybdenum per molecule, as well as variable numbers of iron atoms, and is composed of two pairs of polypeptide subunits. The smaller protein (Fe) consists of two identical subunits as well as four iron atoms per molecule. In both the "MoFe" and "Fe" proteins the iron atoms are accompanied by approximately equal numbers of sulphur atoms. The molecular weights of the "MoFe" and "Fe" proteins have been the subject of conflicting reports but on average they are about 220,000 - 245,000 and 56,000 - 73,000 respectively (Mortenson and Thorneley, 1979). Both "MoFe" and "Fe" proteins are oxygen sensitive, although the "Fe" protein is particularly susceptible. Nitrogenase requires Mg^{++} ions for its activity and, while functional, converts ATP to ADP. Although nitrogenase will convert N_2 to NH_3 it is relatively non-specific in its substrate profile since it will reduce other small molecules which contain triple bonds e.g. acetylene, hydrogen cyanide, hydrogen azide and nitrous oxide. This ability of nitrogenase to reduce acetylene is the basis of the acetylene reduction assay for nitrogen fixing ability and this test is a rapid and sensitive method of detecting the activity of nitrogenase in whole cells or in cell extracts. A "side reaction"

of nitrogenase is the ability to reduce protons (H^+) to hydrogen gas and bioenergetically, the use of nitrogenase to produce hydrogen is a wasteful process because of the substantial energy demands of the enzyme. (Winter and Burris, 1976; Shanmugan *et. al.*, 1978; Mortenson and Thorneley, 1979). The fact that nitrogenase activity is oxygen-labile and energy dependent has serious implications for the hopes of transferring, and effecting expression of, nitrogen fixation genes in higher plants (see page 20).

2. The regulation of nitrogen fixation

It may be expected that a process which is highly energy demanding, such as nitrogen fixation, would be subject to delicate regulatory controls, and indeed this is the case in Klebsiella. The NH_3 produced by nitrogenase is converted to glutamate by the enzymes glutamate synthetase (G.S.) and glutamate synthase (G.O.G.A.T.), as depicted in reactions (2) and (3) (Nagatani *et. al.*, 1971). The synthesis of nitrogenase is repressed by



amino-acids or NH_4^+ (Parajko and Wilson, 1970). Glutamate can also be formed by the action of the enzyme glutamate dehydrogenase (G.D.H.) on ketoglutarate as depicted in reaction (4).



Studies with Klebsiella and E. coli have shown that when fixed nitrogen is abundant reaction (4) is the predominant pathway of glutamate formation, due to the high K_m of GDH for 2 Ketoglutarate or NH_3 . Conversely, at low fixed nitrogen availability, the high K_m of GDH for such substrates makes the reaction (4) unfavourable and the reactions mediated by G.S. and G.O.G.A.T. become more favourable. This fact allows the cell to function in times of fixed nitrogen depletion although, because ATP is consumed in the G.S./G.O.G.A.T. pathway, it is at the expense of an energy drain on the cell (for review see Tyler,

1978). The enzyme G.S. plays a significant role in the regulation of the cellular nitrogen balance. G.S. is subject to a complex pattern of feedback inhibition and its enzymic and regulatory properties are affected by the state of adenylylation of the enzyme (Kingdon *et. al.*, 1967). G.S. is a dodecamer of identical subunits of 50,000 molecular weight. The magnesium dependent catalytic activity of G.S. is inversely preportional to the number of AMP moieties present (0 to 12) and under NH_4^+ depletion the enzyme becomes highly deadenylylated and therefore catalytically active. In times of NH_4^+ excess the enzyme is highly adenylylated and correspondingly enzymically inactive. Hence the NH_4^+ level of the cell determines, indirectly, the enzymic rate of glutamate formation whether it is via the G.D.H. or G.S./G.O.G.A.T. pathways. The actual physiology of the control of NH_4^+ assimilation and the central role of GS in this control is very complex and will not be discussed here (for review see Tyler, 1978).

From even this cursory review of the role of G.S. in the physiology of nitrogen it would be expected that the synthesis and expression of nitrogenase would be related to G.S. activity; this is found to be so. In Klebsiella mutants which are constitutive glutamine synthetase negative (gln), nitrogenase is expressed even under NH_3 excess. Catalytically active G.S. is assumed to "switch on" the nitrogen fixation (Nif) genes although the exact mechanism of this nitrogenase derepression is not fully understood (Streicher, *et. al.*, 1974; Tubb, 1974; Shanmugan *et. al.*, 1978). There is also some recent evidence (Eady *et. al.*, 1978) that O_2 , which can physically inactivate nitrogenase, is also capable of repressing the synthesis of nitrogenase (St. John *et. al.*, 1974). However, it is thought that O_2 repression of nitrogenase may act independently of G.S. (Eady *et. al.*, 1978) but may act via a product of either nif A, nif L or nif E (see pages 7-9). Simplistically then, the regulation of nitrogenase can be regarded as follows. In times of fixed nitrogen excess, NH_4^+

repression of nitrogenase is operating; there is no physiological need for nitrogenase and the derepression effects of catalytically active G.S. are not present. In times of fixed nitrogen depletion, G.S. is catalytically active; NH_4^+ repression is absent and G.S. mediated nitrogenase derepression will occur if oxygen is absent.

3. A summary of the genetics and molecular biology of nitrogen fixation in *Klebsiella*

Transductional analysis using P1 has shown that several nif mutations are linked to his in *K. pneumoniae* (Streicher et. al., 1972) and transfer of this his - nif region of *K. pneumoniae* to *E. coli* produced nitrogen fixing hybrids (Dixon and Postgate, 1972). A variety of nif mutants have been isolated in *Klebsiella* and the mutations responsible for the Nif⁻ phenotype have been mapped by P1 transduction and complementation analysis (e.g. see St. John et. al., 1975; Dixon et. al., 1977; Kennedy, 1977). Merrick et. al. (1978) identified eleven nif genes in *Klebsiella* and Elmerich et. al. (1978) identified twelve nif genes which are arranged into six transcriptional units. However, recently, MacNeil et. al. (1978) identified fourteen nif genes which are arranged into seven operons and the products of nine of these genes have now been identified by 2D polyacrylamide gel electrophoresis (Roberts et. al., 1978). More recently still a fifteenth nif gene has been identified (nif U) (see Cannon et. al., 1979; Riedel et. al., 1979). Using complementation analysis of Mu-induced, frameshift, amber and deletion mutations MacNeil et. al. (1978) found that, of the seven transcriptional units in the Nif cluster, five are polycistronic and two are monocistronic. The transcriptional units and functions are depicted in Figure 1.1. To date all but one (nif J) of the known nif genes of *Klebsiella pneumoniae* have been cloned on small amplifiable plasmids and one such plasmid (pSA30) contains the three known structural genes for nitrogenase (Cannon et. al., 1979). Also, a physical map of the Nif gene cluster

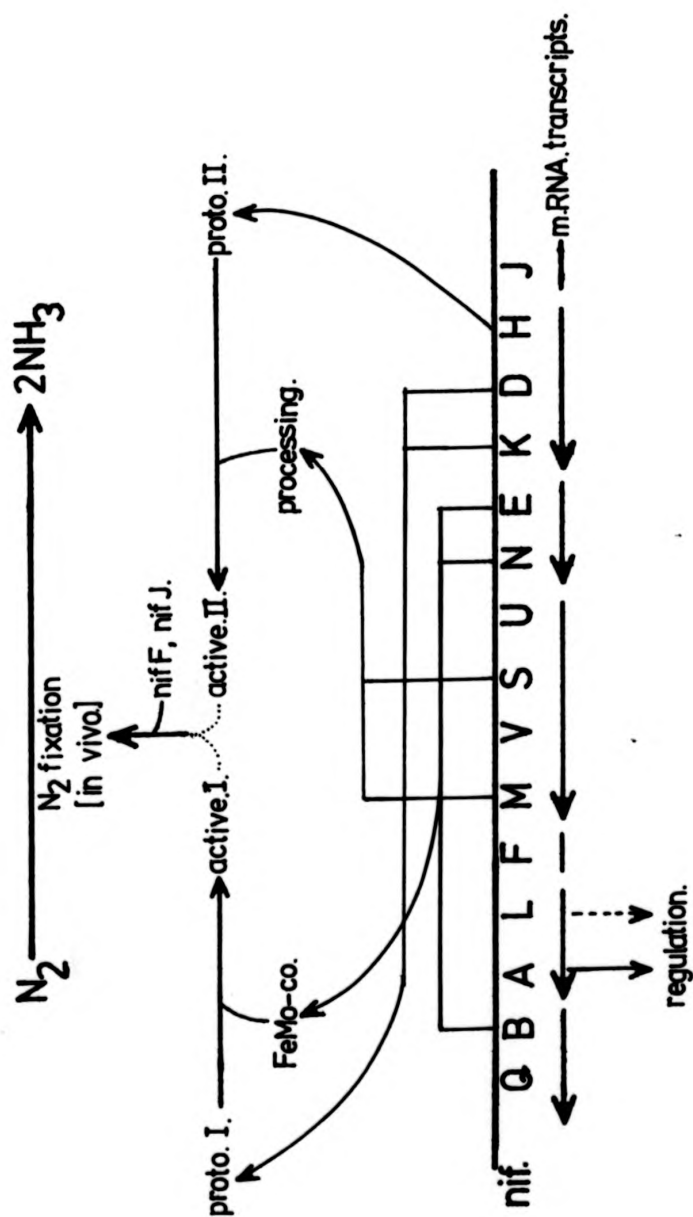


Fig.11 The organisation & function of *nif* genes in *K. pneumoniae*, [after Roberts et al., 1978]

has been made by restriction enzyme analysis (Riedel et. al., 1979). Although it is known that nitrogenase is coded for by the three structural genes nif H, D and K, and although the nif A product has been implicated in the regulation of nif (Dixon et. al., 1977; Roberts et. al., 1978) the regulation of the seven nif operons is still not understood.

Obviously the overall control of the total nif operon cluster is likely to be a complex process, and a great deal remains to be discovered. However, even to date, a marriage of biochemical techniques, classical bacterial genetics and gene cloning methods has helped to create a very detailed knowledge of the Nif genes in Klebsiella pneumoniae.

B). Asymbiotic nitrogen fixation in Rhizobium

1. The Parker-Dilworth hypothesis and breakthrough 1: Free living rhizobial nitrogen fixation.

Over the course of many years a variety of attempts were made by various workers to define the conditions for asymbiotic nitrogen fixation in Rhizobium. All such attempts failed and theories were presented to explain the plant-dependence of rhizobial nitrogen fixation. Ultimately a hypothesis was presented by Dilworth and Parker (Dilworth and Parker, 1969) in which the total genetic information required for effective nitrogen fixation was shared between the plant and the rhizobial endosymbiont. Therefore, neither macrosymbiont nor microsymbiont alone was capable of nitrogen fixation but, in intimate association, would have the total genetic and physiological apparatus for fixation. Such a model was prevalent for several years (e.g. see Tubb, 1974). However later it was discovered that certain rhizobia could fix nitrogen, as determined by the acetylene reduction assay, in loose association with tissue cultures of soya bean (Child, 1975). Subsequently, it was discovered that certain rhizobia could fix nitrogen in loose association with a tissue culture

of a non-leguminous plant such as tobacco (Scowcroft and Gibson, 1975). This suggested that the rhizobia possessed the necessary genetic information for nitrogen fixation and that the physiological condition required for effective fixation did not need to be provided by a legume but was relatively non-specific. Finally it was demonstrated that certain rhizobia were capable of fixing nitrogen in the absence of any plant association (Kurz and LaRue, 1975; McComb et. al., 1975,; Pagan et. al., 1975) and the necessary conditions for asymbiotic rhizobial nitrogen fixation did not prove to be exacting at all! In summary, for asymbiotic nitrogen fixation, the rhizobia required a low O_2 tension; low levels of ammonia e.g. glutamate as a nitrogen "primer" source; a pentose sugar and a dicarboxylic acid such as succinate.

So far this ability of asymbiotic nitrogen fixation in Rhizobium seems to be restricted to the "slow growing" species e.g. Rhizobium "cowpea" 32H1, and so it is clear that, at least for such species, all of the nif genes are present in the bacterial cell. Consequently the slow growing rhizobia can be regarded as slow growing free-living nitrogen fixers which happen to have the ability to enter into symbiosis with legumes. Whether or not conditions can be found which will allow the "fast growing" rhizobia to fix nitrogen asymbiotically is a question for the future.

2. Control of nitrogen assimilation in Rhizobium

It has been suggested (Shanmugan et. al. 1978) that a variety of shifts in rhizobial metabolism occur during nitrogen fixation; including nitrogenase synthesis; supply of ATP and reductant and changes in the pattern of cytochrome synthesis resulting in the formation of a new respiratory chain which is functional in low O_2 tension. Therefore the act of nitrogen fixation in the rhizobia is accompanied by a whole series of physiological activities. It is interesting then that, in nitrogen fixation by free living R.

japonicum, > 94% of fixed nitrogen is exported from the cell (O'Gara and Shanmugan, 1976) and studies with bacteroids yield similar results (Bergersen and Turner, 1967). Since nitrogenase function involves considerable energy demands and is only derepressed in low NH_4^+ availability, it seems ridiculous that the fixing cell should export this newly formed NH_4^+ which it has made at the expense of ATP. In R. "cowpea" 32H1 the G.D.H. pathway of NH_4^+ assimilation is totally absent although a catabolically active G.D.H. is present. Also, although the G.S./G.O.G.A.T. pathway is operative it appears to function at a slow rate and under the strict negative control of NH_4^+ itself! Superimposed on this negative control of the G.S./G.O.G.A.T. pathway by NH_4^+ , the activity of rhizobial G.S. is modulated by repression/depression as well as a reversible adenylation (Ludwig, 1978). It seems ridiculous that, although 32H1 has no alternative method of nitrogen assimilation from the G.S./G.O.G.A.T. pathway, this pathway is subject to such intricate controls. Therefore symbiotic, and presumably free living rhizobia (32H1) act as NH_3 exporters even although this can be a great energetic drain on the cells. Conversely NH_3 assimilation by Rhizobium is restricted and it has been suggested that this reflects the evolutionary commitment of Rhizobium to the symbiotic mode of life, where it attempts to maximise NH_4^+ export to the plant (Ludwig, 1978). It is possible then to envisage the rhizobial bacteroid as nothing more than a fixed nitrogen "factory" for the plant, in symbiosis. The plant supplies photosynthate and provides a low O_2 tension, to protect the nitrogenase (see page 16) and activate bacterial oxidative phosphorylation; the bacteroid exports NH_4^+ and this is presumably converted by plant G.D.H., G.S. and G.O.G.A.T. (Brown and Dilworth, 1975; Robertson et. al., 1975) into glutamate or glutamine. If this is so, that Rhizobium has no alternative method of NH_4^+ assimilation, then it must be considered that the organism is committed, in an evolutionary sense, to a symbiotic "dead-end".

Breakthrough 2: recombination in *Rhizobium*

As mentioned earlier, the molecular biological studies of nitrogen fixation in *Klebsiella* have been particularly elegant and have yielded considerable amounts of detailed information about the organisation and expression of the *nif* genes. This advanced knowledge of the *nif* cluster in *Klebsiella* has been gained from molecular genetic and physiological analysis of mutants which are defective in nitrogen fixation. The ability to generate and genetically map such mutations is therefore a key aspect of symbiotic nitrogen fixation research. Unfortunately, until recently, it was impossible to determine where in the *Rhizobium* genome any particular mutation lay because no method of detecting rhizobial genetic exchange existed. Prior to 1976 there had been no unequivocal demonstration of chromosomal gene transfer, by conjugation, in bacteria unambiguously identified as *Rhizobium* yet conjugational transfer of R-factors had proved possible both into *Rhizobium* and between rhizobia (Beringer, 1974; Cole and Elkan, 1973; Datta *et. al.*, 1971). In 1976, though, chromosomal gene transfer was finally demonstrated in *R. leguminosarum* (Beringer and Hopwood, 1976) using the P-group plasmid R68.45. Subsequent research using recombinational analysis, has allowed the construction of genetic maps and rhizobial chromosomal circularity has been proved (Johnston and Beringer, 1977; Kondorosi *et. al.*; 1977; Meade and Signer, 1977). Details of rhizobial recombination are presented later (see section on the genetics of *Rhizobium*). However it is pertinent to say at this stage that the contribution of R-factor mediated recombination studies to rhizobial genetic research cannot be overestimated. Such carefully controlled genetic experiments have both highlighted the inadequacies of earlier reports (see table 1.1) and allowed a basic genetic analysis of the rhizobial genome.

The *Rhizobium*/legume interaction

There are several steps in the development of a successful

symbiosis, namely:

1. Partner recognition.
2. Root hair infection.
3. Rhizobial migration into the cortex.
4. Bacterium - bacteroid transition and nitrogen fixation.

1. Partner recognition

For many years it has been known that each species of Rhizobium will only enter into a symbiotic relationship with specific legumes. These plant-bacterium combinations are called "cross-inoculation groups" e.g. clovers (Trifolium spp.) are only nodulated by R. trifolii and R. trifolii can only nodulate clovers. What determines the specificity of this interaction is unknown but recent reports suggest that plant lectins play a vital role in this recognition of partners (Dazzo and Hubbel, 1975; Dazzo and Brill, 1977) and the specificity of plant/bacterium interaction may be a reflection of the specificity of the plant lectin/bacterial exopolysaccharide complex, (Sanders et. al., 1978). Sanders et. al. (1978) showed that mutants of R. leguminosarum, which were defective in the synthesis of extracellular polysaccharide, failed to nodulate peas whereas the mucoid parent of such mutants was symbiotically effective. Spontaneous mucoid revertants, of these non-mucoid mutants, regained the ability to nodulate the pea host. Therefore it seems that the exopolysaccharide is essential for nodulation of the pea by R. leguminosarum.

In one case a legume lectin has been purified from white clover seeds and seedling roots. This recognition protein has been called Trifoliin and it acts as a specific agglutinin, of R. trifolii cells, even at low concentrations (Dazzo et. al., 1978). However the exact roles of bacterial exopolysaccharide and legume lectins in the partner recognition process have still to be determined.

2. Root hair infection

After recognition of symbiotic partners the next stage in the

formation of an effective symbiosis is the infection of the plant root hairs by the rhizobia. Various studies of ineffectiveness have been conducted using mutants selected as auxotrophs, or as resistant to phages or antimetabolites (e.g. see Kleczkowska, 1950; Schwinghamer, 1964; Staphorst and Strijdom, 1971; Scherrer and Denarie, 1971; Denarie et. al., 1975; Pankhurst and Craig, 1979). Based on studies with various mutants several chemicals have been cited as important in the infection process e.g. indole acetic acid (Higashi et. al., 1971); cytokinins (Phillips and Torrey, 1970) and pectic enzymes (Hubbell, 1970). However the physiology of the infection process is still poorly understood. Some, but not all, purine auxotrophs prove to be ineffective (Schwinghamer, 1969; Scherrer and Denarie, 1971). Similarly, Denarie et. al. (1975) found that reversion of ineffective leu, ura, ilv or ade mutants to prototrophy restored effectiveness. In some cases the addition of the specific auxotrophic requirement to the plant allowed effective nodulation by an auxotrophic microsymbiont e.g. the addition of riboflavine to the plant infected by a riboflavine mutant of R. trifolii allowed effective nodulation (Schwinghamer, 1970; Pankhurst et. al., 1972). However there can be variable results from different plant/bacterial associations. Schwinghamer (1969) found that a pur strain of R. leguminosarum could not nodulate one variety of pea but formed ineffective nodules on another strain of the same legume.

3. Rhizobial migration into the cortex

In response to the bacterial invasion the plant produces cellulose tubes through which the rhizobial cells migrate. These tubes are called infection threads and the plant mechanism responsible for the morphogenesis of such threads is unknown (Li and Hubbell, 1969). However, several investigations of the biogenesis of such infection threads have been conducted using genetically marked bacterial mutants. Pankhurst (1974) found that, using antimetabolite resistant mutants, a class of mutant could be isolated which failed to induce infection

threads within the nodule. Such mutants had altered cell envelopes, suggesting that the bacterial envelope is important for nodulation. Similarly all viomycin resistant mutants prove to be ineffective (Schwinghamer, 1964; Hendry and Jordan, 1969) and viomycin resistance is due to an accumulation of phospholipid with a change in the mutants ability to exchange cations in the cell envelope (Mackenzie and Jordan, 1970; Yu and Jordan, 1971). Recently Pankhurst and Craig (1979) have shown that mutants of R. "cowpea" 32F1 which were selected as 2 and 3 step D-cycloserine resistant mutants were capable of forming infection threads but, in most cases, the rhizobia were not released from the infection threads to form bacteroids. Pankhurst and Craig suggested that cell wall permeability changes, resulting in D-cycloserine resistance, were responsible for the phenotypes.

4. Bacterium - bacteroid transition and nitrogen fixation

When the bacteria are released from the infection threads, into the cortical cells, they differentiate into spheroplast-like forms called bacteroids and it is the bacteroid which is the vehicle of nitrogen fixation (Nutman, 1977). In conjunction with the genesis of bacteroids the plant produces a rapid cell growth and deposition around the bacteroids to supply the bacteroid-dense zone with vascular tissue. Studies on ineffective mutants have shown that in some cases the block in the ability to form bacteroids can be reversible e.g. as mentioned before, Pankhurst et. al. (1972) showed that a riboflavine auxotroph of R. trifolii could enter into effective symbiosis with the plant if riboflavine was provided but if no riboflavine was added the Rhizobium failed to develop into bacteroids. Pankhurst (1974) also isolated antimetabolite resistant ineffective mutants which produced pleomorphic bacteroids which were larger than wild type. More recently Pankhurst and Craig (1979) isolated several classes of D-cycloserine resistant mutants including classes which either failed to generate bacteroids or did so very poorly. Changes in the cell wall/ cell membrane complex

are thought to occur as the rhizobia are transformed from bacteria to bacteroids (Bergersen, 1974, van Brussel et. al., 1977). However to date the physiology of the bacterium/bacteroid transition is ill understood.

When in the bacteroid state the rhizobia are capable of fixing nitrogen by the action of nitrogenase. However, as mentioned earlier, nitrogenase is particularly O_2 - sensitive. To circumvent this problem the molecule leghaemoglobin is synthesised in the nodule, as a co-operative act between plant and bacterium. The apoprotein of leghaemoglobin is synthesised by the legume and the haeme moiety by the bacterial endosymbiont (e.g. see Dilworth, 1969; Cutting and Schulman, 1969; 1971; Broughton and Dilworth, 1971; Verma et. al., 1974). Leghaemoglobin acts as a "sink" for O_2 molecules thereby allowing the synthesis and catalytic activity of nitrogenase (Truchet, 1972; Bergersen and Goodchild, 1973; Gourret and Fernandez-Arias, 1974). It appears that the optimum O_2 concentration for nitrogenase activity, near $0.1 \mu M$ free O_2 (Bergersen and Turner, 1975), coincides with the O_2 requirement for optimum ATP production in R. japonicum. Hence the O_2 - regulatory effects of leghaemoglobin in the nodule act at the level of O_2 - protection of nitrogenase but also at the level of regulation of the ATP provision for nitrogenase. Studies with D-cycloserine resistant mutants have also suggested that changes in the rhizobial cell wall affect the bacteroid ability to develop nitrogenase activity (Pankhurst and Craig, 1979). Also, recent studies with mutants demonstrate that nitrogenase and nitrate reductase do not share the same molybdenum cofactor (Kiss et. al., 1979).

General considerations of symbiotic development

Overall the studies of the relative contributions, to the symbiosis, of the genetic background of the bacterium and of the plant (for reviews see Nutman, 1955; 1969) have been inconclusive. However the developmental process of symbiotic nitrogen fixation is obviously very

complex. Nevertheless, the fact that Rhizobium can fix nitrogen in association with non-leguminous tissue cultures (Scowcroft and Gibson, 1975) and asymbiotically (Keister, 1975; Pagan et. al., 1975; Kurz and LaRue, 1975; McComb et. al; 1975) suggests that the main roles of the plant in symbiosis are the provision of leghaemoglobin apoprotein for the leghaemoglobin dependent regulation of O_2 access to the bacteroid; the supply of photosynthate as a bacteroid energy source and, finally, the construction of an intricate vascular system for both the transport of photosynthate and export of the fixed nitrogen. A recent report (Legocki and Verma, 1979) shows that although effective nodulation requires the expression of both host and bacterial (Maier and Brill, 1976) genes, at least one protein can be detected which is specifically synthesised in nodules during nodulation and is not made by either the R. japonicum or soy bean symbionts in isolation. This protein is called Nodulin-35 and it is suggested that the plant may make this protein under the direction of Rhizobium (Legocki and Verma, 1979). The possibility that there are a variety of nodule-specific plant proteins (Nodulins) under rhizobial control is an exciting prospect for future studies of the symbiotic relationship.

Potential developments in genetic engineering of nitrogen fixation

Before any significant developments in the construction of agriculturally useful, nitrogen fixing plants can be achieved, a great deal more information has to be discovered about the basic genetics and biochemistry of the rhizobia and plant host. Until a reasonable level of knowledge is achieved in such areas no important practical advances are liable to be made in the production of agriculturally important food crops by the methods of in vivo genetic breeding and "genetic engineering".

Over the past few years the new biotechnology of "genetic engineering" has been alluded to as a panacea for such diverse problems

as the inborn errors of human metabolism and low food production. In almost all debates on the potential benefits and hazards of genetic engineering the ability to increase global biological nitrogen fixation is cited as one of the most important future developments likely to be made possible through the new technology. The implication is that a scientific "breakthrough" of this type would solve the food problems of the starving and undernourished of the third world countries (e.g. see Hardy and Havelka, 1975; Evans and Barber, 1977; Child, 1976). I believe that this attitude is naive and dangerous, both scientifically and politically (see pages 20 and 309).

There are several potential approaches for increasing biological nitrogen fixation but all approaches have associated problems (Evans and Barber, 1977). The first type of approach to increasing biological nitrogen fixation is to attempt to increase the efficiency of the existing biological process. As mentioned earlier a considerable supply of ATP is required for the catalytic activity of nitrogenase and a significant proportion of this energy is "lost" to the cell in the nitrogenase-dependent evolution of H_2 from protons (Evans et. al., 1977). However some strains of Rhizobium japonicum possess a unidirectional hydrogenase which can oxidise hydrogen but cannot lead to hydrogen evolution. This hydrogenase allows the hydrogen formed by nitrogenase to be recycled, with the concomitant regeneration of ATP, and protects the nitrogenase from oxidative damage (Dixon, 1972; Evans et. al., 1977). Hence the recycling of hydrogen can help to "conserve" ATP for the fixation of nitrogen. Recently, mutants of R. japonicum which are defective in hydrogen uptake (Hup^-) have been isolated (Maier et. al., 1978) and studies on the efficiency of nitrogen fixation in such strains will help to determine the extent to which the genetic provision of a unidirectional hydrogenase could enhance the biological productivity of nitrogen fixing organisms.

Other factors which limit nitrogen fixation by the root nodule

symbiosis include environmental parameters such as water, light or CO₂ supply and to a certain extent these aspects can be improved to an optimum level. However another intrinsic limitation to nitrogen fixation is the efficiency and quality of photosynthate supply to the bacteroid (Hardy and Havelka, 1975). Again, the provision of photosynthate is subject to genetic and environmental controls (Hardy, 1977; Bethlenfalvay and Philips, 1977) and so can be improved, up to a point, to allow greater provision for nitrogenase function.

The major hope for increasing the global nitrogen fixation level comes from the research involving genetic engineering of the Nif genes; both between prokaryotes and from prokaryotes to agriculturally important eukaryotes (Shanmugan and Valentine, 1975). The basis of this theoretical possibility is that if the genetic capability for nitrogenase synthesis could be introduced, maintained and expressed in crop plants then the plant would have no need to enter into symbiosis, for nitrogen fixation. Ultimately, it is hoped, it may be possible to introduce Nif genes into cereal plants and thereby eliminate the need for nitrogenous fertilizer to enhance the crop productivity. Theoretically it may be possible to introduce the Nif genes into plant cells using the Ti plasmid of Agrobacterium as a vector (Schell and Van Montagu, 1977) or by using a natural plant virus, such as cauliflower mosaic virus, as the vehicle for Nif transfer (Meagher, 1977). However the introduction of Nif into eukaryotes does not mean that expression of the Nif genes will necessarily occur or, even if expression does occur, that the nitrogenase will be catalytically active (see page 20).

An alternative prospect for the engineering of biological nitrogen fixation is to genetically engineer cereals and other agriculturally important plants in such a way that they will enter into a symbiotic relationship with rhizobia. Obviously leguminous plants, at present, have this genetic capability and identification of the essential

"symbiotic genes" may one day allow the excision and transfer of such determinants into non-symbiotic plants. Alternatively protoplast fusion techniques may allow the creation of hybrid plants, which are part legume and part non-legume, which will enter into symbiosis with Rhizobium (Vasil et. al., 1977).

Problems associated with the genetic manipulation of nitrogen fixation

All of the projected methods of genetic engineering for biological nitrogen fixation have associated technical problems and earlier ideas about the transfer of Nif, from prokaryotes into plants (Shanmugan and Valentine, 1975) appear quite naive when such problems are considered (Marx, 1977).

Although it may be possible to engineer more efficient symbiotic nitrogen fixation, by introduction of hydrogenase for ATP regeneration and by the enhanced supply of photosynthate to the bacteroid, there are reasonable fears that the diversion of photosynthate from plant growth to nitrogenase action may lead to a decreased biological productivity. Consequently an optimum level will be reached, in terms of productivity, beyond which genetic interference with the efficiency of existing symbiotic systems cannot go. It is because of the realisation of this fact that greater hopes for enhanced global nitrogen fixation surround the recombinant DNA techniques of transgenesis. Nevertheless, once again, major problems exist with such techniques.

Although a suitable vector may well be found for the transfer of the prokaryotic Nif gene cluster into plant cells, several other "physiological hurdles" exist before expression could be effected in a plant. The Nif gene cluster may not be transcribed by eukaryotic polymerase since the plant polymerase may not recognise the prokaryotic promoters or terminator sequences (Helinski; 1977). Correspondingly, although transcription may be effective, translation of the various Nif messages may not occur in the eukaryotic cell. Even if the Nif genes are transcribed and translated effectively and nitrogenase is produced,

the enzyme, as discussed earlier is very sensitive to O_2 . Photosynthesising plants generate O_2 and so the O_2 tension in the area of the nitrogenase enzyme would have to be stringently controlled to prevent inactivation of the catalytic activity of the enzyme. Superimposed on this O_2 -lability is the fact that O_2 can actually repress the synthesis of nitrogenase. Hence, in a plant system, the enzyme either has to be compartmentalised, or supplied with some method of chemical sequestration of the O_2 , as a prerequisite for expression.

Another problem for the expression of nitrogenase in plant cells is that nitrogenase activity is very energy dependent and so its' synthesis and expression would act as a significant energy drain on the genetically engineered cells. The possibility that cereal plants could be engineered to allow them to enter into symbiotic relationship with nitrogen fixing organisms, is dependent on sophisticated in vivo and in vitro genetic manipulation and thus would necessarily be dependent on a vast background of genetic information about the cereals. Unfortunately, to date, the genetics of such plants is not sufficiently advanced to allow such sophisticated genetic engineering. Finally there has been some concern that the gradual elevation of global biological nitrogen fixation levels will lead to an ecological imbalance in the ammonia in the biosphere. Ammonia can have deleterious ecological effects (Marx, 1977); hence the concern about excessive ammonia production by widely distributed nitrogen fixation. However, because of the many constraints on effective nitrogenase activity, as mentioned above, this ecological concern can be effectively disregarded.

It seems likely, therefore, that the immediate prospects for genetic engineering of biological nitrogen fixation are not great, and that earlier hopes were scientifically naive. However the scientific approach as a solution to the world food problem is also politically naive and this will be discussed later. (See post script).

THE GENETICS OF RHIZOBIUM

There have been many reports of genetic exchange in the rhizobia. All of the three known methods of genetic transfer; namely transformation, conjugation and transduction, have been reported as occurring in Rhizobium. Unfortunately, the vast majority of these reports have suffered from similar shortcomings in that they show an embarrassing absence of the standard genetical controls e.g. in a transformation assay; sterility of DNA preparation; reversion frequencies and DNA^{ase} sensitivity of transforming activity. Even in reports where such controls have been included there may have been no attempts at characterisation of the putative "transformants", "transductants" or "transconjugants". Bearing in mind that rhizobial cultures are easily contaminated and overgrown, as mentioned earlier, then characterisation of the colonies produced by these genetic experiments is of crucial importance to define the bacteria as rhizobia. Because of the low standard of such genetic experiments an area of mythology and sciolism had arisen in rhizobial research, prior to 1976, regarding what could and what could not be achieved genetically with these bacteria. This criticism of the general standards of rhizobial genetics is not sheer pedantry because the rigour with which one can demonstrate genetic exchange is essential to the future reproducibility of rhizobial genetics and, therefore, its advance. Other workers have made similar criticisms (e.g. Beringer, 1974) although the criticism of the state of rhizobial genetics has never really manifested itself openly in recent publications. As a guide to the shortcomings of most of the rhizobial genetics publications up to 1976 a table has been compiled which highlights some essential feature of each genetic experiment which was inadequately controlled (see Table 1.1)

Table 1.1

Summary of major pre-1976 papers on rhizobial genetics

a) Transformation reports

REFERENCE	MARKERS(s) TRANSFERRED	STERILITY OF DNA	SENSITIVITY TO ase DNA	SPONTANEOUS MUTATION	CHARACTERISATION OF "TRANSFORMANTS"
Balassa(1960)	cys, host range	-	-	-	-
Balassa(1963)	^R str, str dependence, host range, cys, effectiveness	Yes	Yes	Yes	-
Balassa and Gabor(1961)	^R str	Yes	-	Yes	-
Balassa and Gabor(1965)	str dependence	Yes	Yes	Yes	-
Ellis et.al.(1962)	^R Cml, crystal violet tolerance, ^R str	Yes	Yes	Yes	Yes
Gabor(1965)	^R str, independ- ence and depend- ence.	-	Yes	Yes	-
Gadre et.al.(1967)	^R pen, fructose independence	Yes	Yes	Yes	-
Higashi(1967)	host range	-	-	Yes	Yes
Humphrey and Vincent(1963)	virulence	Yes	-	-	Yes
Izraelski(1957)	host range	Yes	-	Yes	Yes
Kleczkowska(1961)	ineffective- ness	-	-	-	-
Kleczkowska(1962)	"	-	-	-	Yes

a) Transformation reports (Contd.)

REFERENCE	MARKERS(s) TRANSFERRED	STERILITY OF DNA	SENSITIVITY TO DNAase	SPONTANEOUS MUTATION	CHARACTERISATION OF "TRANSFORMANTS"
Kleczkowska(1963)	ineffective- ness	-	-	-	Yes
Kleczkowska(1964)	"	-	-	-	-
Kleczkowska(1965)	ineffective- ness and effect- iveness	-	-	Yes	Yes
Klein and Klein(1953)	tumour formation	-	-	-	-
Lange and Alexander(1961)	host range	-	Yes	Yes	-
Ljunggren(1960)	effectiveness	-	-	-	-
Mareckova(1969)	str ^R	-	-	Yes	-
Mareckova(1970)	virulence	-	-	Yes	Yes
Raina and Modi(1969)	ade	Yes	Yes	Yes	-
Raina and Modi(1971)	gelatinase	-	-	-	-
Raina and Modi(1972)	str ^R , ile, leu ura ^R	-	-	-	-
Sen et.al.(1969)	str ^R , crystal violet resistance	-	Yes	Yes	Yes
Sik and Orosz(1970)	phage DNA	-	-	N.A.	N.A.
Sik and Orosz(1971)	"	-	-	N.A.	N.A.
Staniewski et.al.(1971)	"	-	-	N.A.	N.A.
Yamane and Higashi(1963)	host range	-	Yes	Yes	-

a) Transformation reports (Contd.)

REFERENCE	MARKER(s) TRANSFERRED	STERILITY OF DNA	SENSITIVITY TO ase DNA	SPONTANEOUS MUTATION	CHARACTERISATION OF "H ⁺ -TRANSFORMANTS"
Zelazna(1963)	<u>str</u> ^R	Yes	Yes	Yes	-
Zelazna(1964a)	<u>str</u> ^R	-	-	-	-
Zelazna(1964b)	<u>str</u> ^R	-	-	-	-
Zelazna-Kowalska <u>et. al.</u> (1971)	<u>str</u> ^R , colony morphology	-	-	-	-
Zelazna-Kowalska and Lorkiewicz(1971a)	<u>str</u> ^R	-	-	-	-
Zelazna-Kowalska and Lorkiewicz(1971b)	<u>str</u> ^R	-	-	-	-

Yes = control present

- = control absent

N.A. = not applicable

b) Conjugation reports

REFERENCE	MARKER(S) TRANSFERRED	SPONTANEOUS MUTATION	CHARACTERISATION OF "TRANSCONJUGANTS"
Bose and Venkataraman(1969)	<u>str</u> ^R , <u>pen</u> ^R	-	-
Higashi(1967)	host range	Yes	Yes
Kaushik and Venkataraman(1972)	<u>str</u> ^R , u.v. ^R	-	-
Lorkiewicz <u>et.al.</u> (1971)	<u>str</u> ^R , auxotrophies	-	-
Heumann(1968)	auxotrophies and pigment- ation	-	Yes
Heumann <u>et.al.</u> (1970)	*	-	Yes
Puhler and Heumann(1970)	*	-	Yes
Heumann <u>et.al.</u> (1971)	auxotrophies and pigment- ation	-	Yes
Puhler <u>et.al.</u> (1972)	RP4	-	-
Heumann(1972)	auxotrophies	-	Yes
Beringer(1974)	P-group plasmids	Yes	Yes

Yes = control present
 - = control absent
 * = reported in abstract

c) Transduction reports

REFERENCE	Marker(s) transferred	Sterility of phage lysate	Sensitivity to DNAase	Spontaneous Mutation	Characterisation of "transductants"
Kowalski(1966)	<u>str</u> ^R	-	-	-	-
Kowalski(1967)	<u>str</u> ^R	Yes	Yes	Yes	-
Kowalski(1968)	<u>str</u> ^R	-	-	-	-
Kowalski(1969)	<u>str</u> ^R	-	-	-	-
Kowalski(1970)	<u>str</u> ^R	Yes	-	Yes	-
Kowalski(1971)	<u>str</u> ^R , <u>lys</u> , effectiveness	Yes	-	Yes	-
Sik and Orosz(1971)	<u>cys</u>	-	Yes	-	-

Yes = control present

- = control absent

1. Transformation

The majority of the reports of gene transfer in Rhizobium have concerned transformation (see Table 1.1a.) Unfortunately, to date, no unequivocal demonstration of transformation exists for this genus. A variety of markers have been used in transformation experiments with Rhizobium e.g. antibiotic resistance, host range, amino acid dependence, infectiveness and effectiveness. However, one criticism or another can be levelled at all transformation claims in the rhizobia, including the failure to identify the bacteria as rhizobia; failure to quantify reversion frequencies; failure to demonstrate that the DNA preparation was sterile and failure to demonstrate the sensitivity of the "transformation" to DNA^{ase}.

This apparent inability to construct the conditions necessary for the transformation of Rhizobium could prove a problem because, apart from precluding its use as a method of fine-structure mapping, it also means that the use of transformation techniques in rhizobial genetic engineering is not yet possible. The importance of calcium-dependent transformation and transfection techniques in the genetic engineering of E.coli strains cannot be overestimated (Benzinger, 1978). Indeed this importance of transformation highlights the need to develop a transformation system in a genus, such as Rhizobium, which is, arguably, considerably more important than E.coli.

This failure to transform the rhizobia does seem all the more remarkable when one considers that other, relatively closely related genera are now transformable e.g. Agrobacterium (Holsters et.al., 1978) and Pseudomonas (Chakrabarty et.al., 1975; Mylroie et.al., 1977; Gantotti et.al., 1979). Although transformation in Rhizobium has not proved technically possible to date, there are reports of transfection in this genus. Sik and Orosz, (1970; 1971), Staniewski et.al., (1971) and Kondorosi et.al., (1974) have claimed that R.meliloti

can be transfected with phage 16-3 DNA, although either spheroplasts (Staniewski et.al., 1971) or a helper phage (Kondorosi et.al., 1974) have to be used. With either method there is no development of competent cells and the most efficient transfection obtained is low at 10^{-8} . Such publications of transfection are also, unfortunately, subject to the same inadequacies as those pertaining to transformation (see Table 1.1a.). Hence it may prove difficult, or impossible, to find the conditions necessary for the transfection or transformation of Rhizobium.

2. Transduction

The literature on transduction in Rhizobium is far less extensive than that for transformation. Although it has been known for some time that rhizobiophages exist (Laird, 1932) the potential use of phage as a vector for rhizobial DNA has been largely underdeveloped. Until very recently, transduction had only been demonstrated in R.meliloti although such transduction is of both the generalised and restricted forms. Kowalski, (1967; 1970) found several phages which were capable of effecting transfer of streptomycin resistance or lysine independence at frequencies of 10^{-5} to 10^{-8} . Although no evidence of linkage could be demonstrated between lys and effectiveness by such studies, Kowalski and Denarie (1972) were able to show cotransduction of effectiveness and leu in R.meliloti, using ϕ L5. Kowalski's experiments involved generalised transduction in which any segment of the bacterial genome may be transferred to the recipient. Consequently, generalised transduction may be used in the fine structure mapping of any part of the genome, allowing the resolution of closely linked markers (Hayes, 1968). The value of generalised transduction has been demonstrated, in Rhizobium, very recently, both as a method of resolving closely linked genes (Buchanan-Wollaston, 1979; Casadesus and Olivares, 1979) and showing the extra chromosomal

nature of nodulating ability (Johnston et.al., 1978b). Buchanan-Wollaston (1979) used two virulent rhizobiophages (RL38 and RL39) to cotransduce auxotrophic and antibiotic markers as well as two P1 group R plasmids. The efficiency of transduction varied from 10^{-5} - 10^{-6} for auxotrophies to 10^{-7} for R plasmids and interspecific transduction from R.leguminosarum to R.trifolii proved possible, although transduction in the opposite direction could not be detected. This failure to show transduction between R.trifolii as donor and R.leguminosarum as recipient was unlikely to be due to restriction since R68.45 mediated conjugation using the same donor and recipient cells leads to the formation of stable recombinants at a similar frequency to intraspecific R.leguminosarum crosses (Johnson and Beringer, 1977). The effect could not be explained on the basis of reduced adsorption of the transducing phage to the R.leguminosarum recipient because the efficiency of plating on R.leguminosarum was higher than on R.trifolii (Buchanan-Wollaston, 1979).

Because ØRL38 and ØRL39 are virulent phages, the transducing lysates had to be U.V.-irradiated prior to transduction in an attempt to reduce the killing effects of the phages (Buchanan-Wollaston, 1979). This is not the first time that virulent phages have been used as generalised transducing phages. Virulent phages have been used as generalised transducers, using U.V.-irradiated lysates of ØCr30 in Caulobacter (Ely and Johnson, 1977); or suppressible phage mutants of T1 in E.coli (Drexler, 1970) or ØSP1 in B.subtilis (Yasbin and Young, 1974); and finally using temperature sensitive phage mutants of MX4 in Mycococcus xanthus (Campos et.al., 1978). Apart from their ability to act as generalised transducing phages, no more details about ØRL38 and ØRL39 are known.

For fine structure mapping, strain construction and transposon-mediated mutagenesis (Beringer et.al., 1978) the use of generalised transducing phages, such as ØRL38 and ØRL39, will prove particularly

useful in the future. However, for certain purposes, restricted or specialised transducing phages are of more value than their generalised transducing counterparts.

Specialised transducing phages have a variety of uses in molecular biology e.g. the quantitation of specific mRNA's (Imamoto et.al., 1965); transcriptional regulation studies (de Crombrugghe et.al., 1971; Zalkin et.al., 1974); the amplification of specific genes (Muller-Mill et.al., 1968) and the identification of specific gene products (Lutkenhaus and Donachie, 1979). However, the specialised transducing phages are of limited utility in mapping experiments apart from their use as vehicles for deletion mutants (e.g. see Saito and Uchida, 1978) and in complementation tests (e.g. see Lutkenhaus and Donachie, 1979).

To date specialised transduction has been demonstrated in R.meliloti (Sik and Orosz, 1971; Svab et.al., 1973; Svab et.al., 1978) but no other rhizobial species. Sik and Orosz (1971) reported transduction by $\phi 16-3$ of cys at frequencies of 10^{-3} to 10^{-7} and generated H.F.T. lysates which could be used to transduce at frequencies of 10^{-1} - 10^{-2} . However in this earlier report no details of controls were presented. More recently a far more detailed analysis of the $\phi 16-3$ /R.meliloti system has been published (Svab et.al., 1978). It has been shown, by R68.45 mediated recombination mapping, that the chromosomal attachment site of $\phi 16-3$ is close to the cys-46 and met-5 markers. Although phage 16-3 cannot transduce met-5 it is capable of transducing the cys-46 marker at a frequency of 10^{-6} to 10^{-7} . Most (2/3) of the transductants to cys⁺ produced by phage 16-3 were heterogenotes and were unstable for the cys⁺ marker. Such heterogenotes yielded H.F.T. lysates at a frequency of 10^{-2} . Phage 16-3 can also form defective transducing particles (16-3 dc particles) and transductants from such particles, if raised from multiple infection, can give rise to H.F.T. lysates. Transductants

produced by single infection with a 16-3 dc particle could not give rise to H.F.T. lysates. However phage genes, which map towards the middle of the vegetative 16-3 chromosome, could be demonstrated, in such transductants, by marker rescue. Nevertheless, even on superinfection, such transductants did not give rise to H.F.T. lysates. This situation contrasts with other known defective transducing particles e.g. λ dg (Weisberg and Gottesman, 1969). In an attempt to explain this result, Svab *et.al.* (1978) proposed that the dc particles can be integrated into the chromosome but cannot be excised from it, even by superinfecting phage. However, tandem integration of helper and dc particle genomes into the chromosome could lead, on induction, to the integrated helper providing both a functional and a structural effect on the excision and maturation of the defective particle. Alternatively, Svab *et.al.* propose the defective cys⁺ lysogens are a result of double crossovers at preferential sites between the chromosome of the host and the transducing particle genome. This suggestion that there are specific sequences, near the cys-46 region, which enhance recombination is interesting since, although the cys-46 marker is actually further, than met-5, from att-16-3 (as determined by R68.45 mapping) phage 16-3 cannot transduce met-5 yet it can transduce cys-46 with a frequency of 10⁻⁷.

The study of the phage 16-3/R.meliloti system will be of great value to the advance of rhizobial molecular genetics. This is not only because it represents a system which can combine both transductional and conjugational analysis (Svab *et.al.*, 1978) but because it may prove possible to model the development of this system along the lines of the λ /E.coli K12 system; with respect to molecular genetics; selective gene cloning and the *in vitro* construction of useful phage vectors.

Conjugation

As mentioned earlier, there had been no unequivocal demonstration of conjugal chromosomal marker mobilisation in Rhizobium prior to 1976. Earlier publications claimed that conjugation occurred and that chromosomal transfer could be demonstrated but such reports usually lacked adequate controls or failed to prove the rhizobial nature of the putative transconjugants (see Table 1.1b). The reports, post 1973, can be grouped into two classes. The first class of reports are those of the German group who purport to work on R.lupini. Such reports are extensions of the original work of Heumann (1968) and are subject to identical criticisms (see Table 1.1; and below). The second class of reports come from three groups and these latter publications demonstrate, unequivocally, the existence of plasmid-mediated chromosomal gene transfer in a variety of rhizobial species (Beringer and Hopwood, 1976; Meade and Signer, 1976; Kondorosi et.al., 1977).

Since 1973, publications have appeared concerning a continued analysis of conjugation in bacteria classified as R.lupini (Heumann and Springer, 1977; Puhler and Burkardt, 1978). The origin of the strains used in such studies was Heumann (1968) and at no time have these strains yielded a positive response in plant tests against lupins. There are several other facts which argue against these strains being rhizobial in nature. The original strains isolated were rough mutants of an isolate from a lupin nodule (Heumann, 1968). These strains cannot nodulate lupins and no nodulating revertants have ever been isolated. The strains derived from the original isolates all grow on nutrient agar and in nutrient broth. Although some R.meliloti strains can grow on nutrient agar (e.g. strain Rm2011 of Meade and Signer, 1977) it is exceptional to find other rhizobial species capable of growth in such high amino-acid concentrations. These putative R.lupini strains not only grow in

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nutrient broth but they do so at a remarkably rapid growth rate for "slow growing" rhizobia (doubling time of 90 minutes compared with between 3 and 10 hours for most "slow growers"). Also, $RP4^+$ derivatives of such strains exhibit a high resistance to tetracycline e.g. $RP4^+$ strain 20 has an m.i.c. of $64 \mu\text{g/ml}$. (Puhler and Burkardt, 1978) whereas the m.i.c. of $RP4^+$ R.trifolii tends to be about 10-12 $\mu\text{g/ml}$. Finally, the Heumann strains show pigmentation (Heumann, 1968; Heumann et.al., 1971) and this makes it particularly unlikely that these strains are R.lupini. Because of such criticisms it is difficult to assess the importance of the studies of the German group to the field of rhizobial genetics. Consequently, the real relevance of such work will only become clear when it is possible to demonstrate the genetic identity of these strains with other R.lupini isolates. Alternatively, if it becomes possible to classify strains, unequivocally, as Rhizobium without the use of a plant test then Heumann's strains may be identified more rigorously. Although these strains are interesting in that they show merodiploidisation (Heumann and Springer, 1977) and fertility inhibition by $RP4$ (Puhler and Burkardt, 1978) it does seem unlikely, then, that they are pertinent to the general area of rhizobial genetics.

The findings of the other three groups of workers have proved more reasonable in that great similarities have been found for the genetics of different rhizobial strains; well marked strains have been used throughout; all strains nodulate their respective hosts and the experiments are demonstrably repeatable. As mentioned earlier, Beringer and Hopwood (1976) were first to demonstrate chromosomal gene transfer, by conjugation, in Rhizobium. Almost simultaneously Meade and Signer (1976) reported $RP4$ -mediated recombination in R.meliloti. Beringer and Hopwood used a derivative of the P group R-factor R68 (Haas and Holloway, 1976) which had

shown an increased sex-factor activity in Pseudomonas. This derivative of R68 was designated R68.45 and it mobilised chromosomal genes in R.leguminosarum at a frequency of about 10^{-7} . Using re-combinational mapping, in four factor crosses, linkage was established between four auxotrophic markers. It had previously been shown that RP4 was a very poor sex factor in R.leguminosarum (Beringer, 1974) yet it has a sex factor activity in Acinetobacter (Towner and Vivian, 1976; Towner, 1978). Meade and Signer (1976; 1977) showed that RP4 could be used as a sex factor in R.meliloti and Kondorosi et.al. (1977) showed that R68.45 could be used to map R.meliloti with a greater efficiency of chromosome transfer than was detectable in R.leguminosarum.

Subsequently, Beringer et.al. (1978) used R68.45 to promote more extensive analysis of the R.leguminosarum chromosome and circularity was established, as it was for R.meliloti (Meade and Signer, 1977; Kondorosi et.al., 1977). Interspecific recombination was also achieved (Johnston and Beringer, 1977; Johnston et.al., 1978) and R prime derivatives of R68.45 have been produced (Johnston et.al., 1978). The R-primes can be selected by selecting transconjugants in an R68.45 mediated conjugation from R.meliloti to R.leguminosarum. This is because recombination of R.meliloti DNA into R.leguminosarum occurs at very low frequency and so the transconjugants which arise in this type of mating are unstable heterogenotes carrying the relevant R prime (Johnston et.al., 1978). Why this low recombination frequency occurs is unknown but it is dependent on the direction of transfer i.e. using R.leguminosarum as the donor and R.meliloti as the recipient the frequency of recombination is significantly higher (Johnston and Beringer, 1977). However the expression of R.leguminosarum rif and str genes seems to be reduced in R.meliloti (Johnston et.al., 1978). This effect of decreased expression of

genes in heterospecific matings has been more intensively studied by Johnston et.al. (1978a). There are three regions of the Rhizobium chromosome which code for enzymes of tryptophan biosynthesis (Beringer et.al., 1978a; Johnston et.al., 1978a) and Johnston et.al. (1978a) were able to construct strains, carrying each of the three trp gene clusters, as R prime derivatives. Such R prime-trp containing strains of R.leguminosarum were used to transfer the rhizobial trp genes into P.aeruginosa and E.coli recipients which were defective in known genes controlling the synthesis of tryptophan in these species. Complementation was achieved in the P. aeruginosa mutants; R primes pAJ24J1 complemented trp A, B and F; pAJ73J1 complemented trp C and D and pAJ88J1 complemented trp E (Johnston et.al., 1978a). Using this interspecific complementation the biochemical function of the rhizobial genes can be determined e.g. pAJ24 complements the trp F mutation in P.aeruginosa and therefore must be able to code for the enzyme phosphoribosyl anthranilate isomerase (Johnston et.al., 1978a).

Although rhizobial genes are expressed in Pseudomonas, Johnston et.al. (1978a) were unable to detect expression of such rhizobial trp genes in E.coli. By demonstrating transfer of the R-prime-trp plasmids back out of the E.coli into R.leguminosarum, and expression of trp in the rhizobial host, it was proved that all of the rhizobial trp genes had remained intact in the E.coli host. Hence, failure to express the trp genes in E.coli was not due to plasmid instability, but rather was probably due to an inability of the E.coli polymerase to "recognise" the rhizobial promoters. However, a mutant R prime-trp plasmid was isolated (pAJ24J1) which was capable of suppressing the trp A and F markers of E.coli and this mutant plasmid was still capable of complementing rhizobial and pseudomonad trp lesions (Johnston et.al., 1978a). Hence using R primes, dominance and

complementation studies can be performed in rhizobia and related organisms.

Other uses of promiscuous plasmid-mediated conjugation have been published recently. In their studies on the organisation of the R.meliloti chromosome, Casadesus and Olivares (1979) demonstrated that R68.45-mediated recombinational mapping only yields rough linkage measurements. Using the generalised transducing phage, DF2, these workers were able to provide a more accurate linkage map of specific chromosomal regions. Indeed, Buchanan-Wollaston (1979) has provided similar data for R.leguminosarum. Apart from the mapping of auxotrophies and antibiotic resistance markers promiscuous plasmid mediated conjugation has recently been used to map genes involved in nitrate reduction in R.meliloti (using R68.45)(Kiss *et.al.*, 1979). Stanley and Dunican (1979) have also very recently used RP1 to transfer nif genes from R.trifolii to Agrobacterium and Klebsiella and nitrogen fixing recipients were produced, (see page 46).

Finally, there have been two reports on RP4 derivatives, constructed *in vitro*, containing rhizobial DNA. Jacob *et.al.*, (1976) cloned R.leguminosarum DNA into EcoR1 digested RP4 plasmid DNA. However the rhizobial strains carrying such in-vitro constructed R-primes were unable to mobilise the resident chromosome more frequently than the RP4 parent plasmid. Because of this result Jacob *et.al.* concluded that homology between the plasmid and the chromosome was not the limiting factor in chromosome mobilisation. Interestingly, Johnston *et.al.*, (1978) were also unable to mobilise the chromosome of R. leguminosarum with the R primes produced *in vivo*. In total contrast to these reports, Julliot and Boistard, (1979) were able to mobilise the chromosome of R.meliloti using RP4 derivatives carrying EcoR1 and Hind III cloned segments of R.meliloti DNA. Julliot and Boistard were able to use these novel

plasmids to mobilise the chromosome of R.meliloti from a variety of chromosomal loci and this is the first report of directional transfer, in these bacteria, showing a gradient of marker transmission. Such Hfr type rhizobia will be of great utility in the ordering of chromosomal markers, and in strain construction.

This short review of rhizobial conjugation demonstrates that R plasmid mediated conjugation has permitted a considerable genetic knowledge to accumulate over the past few years. Although transformation has not yet proved possible in the rhizobia the combination of generalised and restricted transduction; and the development of plasmid mediated generalised, and directional, chromosome mobilisation will now provide a basis for extensive and elaborate molecular genetic analysis of the rhizobia.

The molecular biology of Rhizobium

1. Phages

There have been many reports on the isolation of rhizobiophages and studies of phage host range (e.g. see Parker and Allen, 1957; Katznelson and Wilson, 1941; Staniewski, 1970; Barnet, 1972 and Atkins, 1973) but there has not been any detailed analysis of rhizobiophage at the molecular level apart from some genetic mapping of ϕ C (Atkins, 1973) and the temperate phage 16-3 (Orosz and Sik, 1970, Sik and Orosz, 1971; Szende, 1971). There is also an isolated example of DNA/DNA hybridisation kinetic analysis of ϕ C and some related phages (Atkins and Avery, 1974) which demonstrated a high percentage of repetitious sequences in these phages. However, apart from these genetic and kinetic analyses of ϕ C and ϕ 16-3, most studies have been concerned with phage morphology, host range, phage typing, and ultra violet or thermal inactivation kinetics.

There has also been a variety of studies on the adsorption properties of phages which form plaques on certain strains designated as rhizobia. The phage 7-7-1 was found to adsorb specifically to the complex flagella of a strain designated as R.lupini H13-3. Similarly a phage tail-like bacteriocin, produced from a strain tentatively designated as R.lupini 16-3 (Lotz and Mayer, 1972) was found to adsorb specifically to the lipopolysaccharide of another strain called R.lupini 16-2 (Pfister and Lodderstaedt, 1977). The strains 16-3 and 16-2, however, are those isolated by Heumann (1968) and, as argued in an earlier section, are unlikely to be rhizobia. Similarly, strain H13-3 has not been unequivocally designated as Rhizobium. Of the many phages used in phage typing, by Staniewski (1968), the phage 1P has been shown to adsorb to the lipopolysaccharide of various R.trifolii strains (Zajac *et.al.*, 1975). The biochemistry of every stage, from adsorption to lytic phage production, has still

to be determined for one rhizobiophage.

However, there are two systems, involving temperate rhizobiophages, which could prove to be particularly useful for the development of advanced genetic engineering and molecular genetic techniques in the rhizobia. Because there is a genetic map for $\phi 16-3$ (Sik and Orosz, 1971; Szende, 1971); because it is also a specialised transducing phage (Svab *et.al.*, 1978) and because the chromosomal attachment site of $\phi 16-3$ in the *R.meliloti* 41 chromosome is known, (Svab *et.al.* 1978) it may prove possible to develop a variety of techniques analogous to those developed for the phage λ /*E.coli* K12 system. Such techniques may include the construction of *att* $\phi 16-3$ -deleted strains to allow transduction of alternative markers to the *cys* marker normally transduced. Using this technique it may be possible to create a variety of specialised transducing derivatives of $\phi 16-3$, as has been done for λ (Schrenk and Weisberg, 1975). Apart from such *in vivo* constructions, it may also be possible to develop $\phi 16-3$ as a cloning vehicle analogous to those λ derivatives already engineered (Murray and Murray, 1974; 1975; Murray *et.al.*, 1977; Williams and Blattner, 1979). The other system which could prove useful in the development of a molecular analysis of *Rhizobium* and rhizobiophage is the one involving two *R.trifolii* strains, Su297 and Su298, and the temperate phages $\phi 1$, $\phi 7$ and $\phi 8$ (Marshall, 1956; Takahashi and Quadling, 1961; Barnet, 1960; Barnet and Vincent, 1970). This system will be discussed in greater detail later (see Chapter 5). However it is pertinent to mention at this stage that a study of this complex system could help to explain many molecular biological phenomena, including phage recombination; phage evolution; cryptic lysogeny; lysogenic conversion and host-controlled-modification.

2. Bacteriocins

Reports exist of rhizobiocin production and the characterisation of such rhizobiocins (Schwinghamer and Belkengren, 1968; Roslycky, 1967; Schwinghamer, 1971; Schwinghamer et.al., 1973; Schwinghamer, 1975). There are also reports of bacteriocin production, and the analysis of such bacteriocins, in several strains tentatively identified as R.lupini (Lotz and Mayer, 1972; Gismann and Lotz, 1975; Pfister and Lodderstaedt, 1977). As mentioned in earlier criticisms, it seems unlikely that these putative R.lupini strains are rhizobial and so the relevance of these studies to rhizobial molecular biology is doubtful.

Two classes of rhizobiocin have been identified; those of high molecular weight and those of low molecular weight. The high molecular weight rhizobiocins are generally defective phage particles (Schwinghamer et.al., 1973) whereas the low molecular weight rhizobiocins (180,000-200,000 daltons) are usually small proteins with slight carbohydrate, lipid or nucleic acid content.

Although the actions of some bacteriocins have been classified as either bacteriocidal or bacteriostatic in Rhizobium the exact mode of action within the rhizobial recipient has not been determined for any rhizobiocin (Schwinghamer, 1975). Until recently there was no information at all about the genetic basis for rhizobiocinogeny. However there is an exciting report of a strain of R.leguminosarum (strain 248) which carries a plasmid that specifies the synthesis of a bacteriocin (Hirsch, 1978). The plasmid (pRLJ1) can be transferred at high frequency (10^{-2}) to other rhizobia and there is some evidence in favour of the hypothesis that this conjugative, bacteriocinogenic plasmid may carry genetic information required for nodulation (Johnston et.al., 1978b; see pages 444). More recently still, Hirsch (1979) investigated bacteriocin production by 97 strains of

R.leguminosarum and identified two types of bacteriocin. These bacteriocins were designated small and medium; the small bacteriocins being able to diffuse through cellophane while the medium bacteriocins could not. Three isolates were found to carry determinants of medium bacteriocin production and such determinants were self-transmissible with frequencies of 10^{-1} to 10^{-2} . Mobilisation of chromosomal genes was associated with the transfer of such bacteriocinogenic plasmids. This data (Hirsch, 1979) suggests that bacteriocinogeny is very widely distributed in R.leguminosarum.

3. Plasmids and transposons

There have been reports of the occurrence and transferability of multiple antibiotic resistance in R.japonicum (Cole and Elkan, 1973) and other rhizobia (Pariiskaya and Garelova, 1976) although the absence of plant test data in these publications must cast doubt on the claim that the strains were rhizobia. It has proved possible to transfer the tumour inducing (Ti) plasmid of Agrobacterium tumefaciens into R.trifolii. ex planta. To achieve this RP⁴ was used, presumably to mobilise the Ti plasmid from the Agrobacterium to the rhizobial recipient. The Ti⁺ rhizobial recipient was capable of expressing the Ti plasmid genes, such as octopine degradation, and could induce tumours on Kalanchoe as well as effectively nodulate the Trifolium host (Hookyaas et.al., 1977). Interestingly, however, although the Ti⁺ transconjugants of R.trifolii strain 5 could nodulate Trifolium pratense they were incapable of nodulating Trifolium parviflorum although the parental Ti⁻ strain could do so. It was found that the Ti⁺ exconjugant had lost a large plasmid which had been in the parental strain (Hookyaas et.al., 1977).

It has also been shown that the bacteriophage Mu could be introduced into R.meliloti using RP⁴::Mu hybrid plasmids. The Mu phage was expressed in the rhizobial recipient although at a reduced

efficiency compared with in E.coli or Pseudomonas (Boucher et.al., 1977).

Plasmid DNA has been isolated directly from Rhizobium using dye buoyant density gradient centrifugation of "cleared" lysates (Tshitenge et.al., 1975; Dunican et.al., 1976). However no large molecular weight plasmids were found by this method, possibly due to the loss of membrane bound plasmids when the chromosomal/membrane complex was removed. A similar phenomenon was found with Ti plasmid studies (Ledeboer et.al., 1976). Using cleared alkaline lysates; alkaline sucrose gradients and dye buoyant density centrifugation, large rhizobial plasmids were detected. The molecular weight of these plasmids ranged from 0.7×10^8 to 4×10^8 daltons (Nuti et.al., 1977). More recently Casse et.al., (1979) screened a variety of bacterial strains for plasmids, including 25 strains of R.meliloti, 22 of which were found to carry at least one large plasmid. The plasmids isolated by Casse et.al. ranged from 9×10^7 to 2×10^8 daltons.

It has been suspected for some time that effective nodulation ability in the rhizobia could be plasmid coded. Evidence in favour of this hypothesis, until recently, was very circumstantial and came from, often controversial, physical and genetic studies (Higashi, 1967; Dunican and Cannon, 1971; Dunican and Tierney, 1974; Sutton, 1974; Zurkowski et.al., 1973; Dunican et.al., 1976). Correspondingly, there is indirect genetic evidence that nodulation ability is not chromosomally determined since, in interspecific matings involving transfer of all sections of the rhizobial chromosome, nodulating ability was not transferred (Johnston et.al., 1978b). Therefore, because of the evidence in favour of an extrachromosomal determinant(s) of host specificity; and because of the presence of large plasmids in the rhizobia it seemed likely that one or several of such plasmids

could code for host specificity proteins.

Two recent reports have proved particularly interesting with respect to the analysis of host specificity determinants. Beringer et.al. (1978) were unable to transfer an RP4::Mu::Tn5 plasmid from E.coli into R.leguminosarum, although the RP4 could be transferred with high efficiency under identical conditions. However, it proved possible to transfer a novel plasmid (pJB4J1) into a variety of Rhizobium species from an E.coli donor. This plasmid (pJB4J1) was formed as a co-integrate of Mu; the kanamycin resistance transposon, Tn5; and another plasmid, pPH1J1 (Hirsch, 1978) which specifies gentamycin resistance, spectinomycin resistance and low level streptomycin resistance. The pJB4J1 (i.e. pPH1J1::Mu::Tn5) plasmid had a reduced ability to become established, in the rhizobia, compared with the parent plasmid pPH1J1 and exconjugants, from crosses, which expressed resistance to one or more drugs could not transfer drug resistance back out of the rhizobial host. The kanamycin resistant exconjugants, when replica plated, yielded random auxotrophs at a frequency of 0.3% and transduction demonstrated the linkage of kan with auxotrophy. Hence the plasmid pJB4J1 can be used as a vehicle for transposon mutagenesis for the creation of mutations in rhizobia, by insertional inactivation (Beringer et.al., 1978). Using insertional inactivation of genes essential for symbiotic effectiveness, it will prove possible to map such genes by the location of the Tn5 determinant thereby reducing the need for many time consuming plant assay tests and so, in this respect, this development will prove to be a great advance in the analysis of the molecular biology of symbiosis. Indeed another report by the same research group (Johnston et.al., 1978b) makes use of this technique to provide strong evidence in favour of the hypothesis that there is a plasmid basis for nodulation ability. A plasmid was constructed, in vivo.

which was a derivative of the conjugative, bacteriocinogenic plasmid pRL1J1, having Tn5 inserted into or close to the gene(s) coding for the bacteriocin. The linkage of this Tn5 with the bacteriocin determinant was demonstrated by ØRL38 co-transduction. An R. leguminosarum strain carrying this plasmid was used as a donor in matings with an ineffective R. leguminosarum as well as in heterospecific matings. Kanamycin resistant transconjugants were purified and tested for nodulation ability on both peas and the respective normal host of the recipient strain. It was found that the transconjugants had become able to nodulate peas and in most cases were also able to fix nitrogen within their "new" host, although the efficiency of both nodulation and nitrogen fixation varied. The transconjugants were simultaneously capable of nodulating their normal macrosymbionts although the efficiency of nodulation and nitrogen fixation was again reduced. Interestingly, the R. phaseoli transconjugant which was capable of nodulating peas best was the least efficient at nodulating beans, perhaps suggesting that some information required for bean nodulation was either suppressed or lost. Hence the study of Johnston et.al. (1978b) provides strong evidence in favour of the idea that plasmids are involved in host specificity. It cannot be said at this stage whether the nodulation capability (or part of it) is carried on the bacteriocinogenic plasmid, pRL1J1, or is merely mobilised by this conjugative plasmid.

The reason for this rather detailed emphasis on the recent work of Beringer and co-workers is that this work is the perfect demonstration of the rapid advances that can be achieved when well controlled genetic experiments are conducted using the powerful techniques of standard molecular genetics. And to a certain extent the progress made by these studies merely highlights the inexcusable inadequacies of earlier rhizobial genetic work (see Table 1.1)!

Finally, recently, Stanley and Dunican (1979) were able to use RPl to mobilise the Rhizobium Nif genes to Agrobacterium and Klebsiella. The high intergeneric co-transfer rates of Nif with RPl suggested co-transfer of a Rhizobium plasmid containing nif genes, and three alternatives exist to explain this effect. It may be that an R' nif plasmid was formed; RPl may have mobilised a naturally occurring rhizobial nif plasmid as a co-integrate or, finally, a resident, conjugative nif plasmid may have been transferred via the RPl conjugative pili.

The prospect that nodulation ability and nitrogen fixation ability are expressions of extrachromosomal determinants in Rhizobium is an exciting one. Construction of recombinant plasmids carrying such rhizobial functions, along with the Ti plasmid of Agrobacterium, may eventually allow the formation of a suitable vector for the introduction of nif genes into agriculturally important plants.

Purpose of this study

At the beginning of this study no unequivocal demonstration of transformation or transduction existed in R.trifolii, and from a molecular biological and genetic viewpoint, the rhizobia were particularly poorly understood. Consequently, this study was undertaken in an attempt to obtain basic information about the distribution of lysogeny and bacteriocinogeny in Rhizobium; and to try to develop a transformation or transduction system in R.trifolii. Also, because of the dearth of molecular biological knowledge in this genus, the Su297/Su298 system was investigated in detail, as a model system for elucidating some of the molecular processes which occur in this genus.

CHAPTER II

GENERAL MATERIALS AND METHODS

Methods and materials for general use are described in this section.
Where relevant, other methods will be described in subsequent chapters.

MEDIA

All media were autoclaved at 15 p.s.i. for 20 minutes.

Yeast Extract Mannitol Broth (Y.M.B) (Vincent, 1970).

MgSO ₄ .7H ₂ O	0.2g
NaCl	0.1g
Mannitol	10.0g
Yeast Extract (Difco)	1.0g
K ₂ HPO ₄	0.5g
Distilled H ₂ O to 1 litre.	
Final pH adjusted to 6.8	

Glucose Salts Yeast Extract Casein Broth (G.S.Y.C.)

(Atkins, 1973 variation of Schwinghamer, 1970).

Yeast Extract (Difco)	1.0g
Casein Hydrolysate (B.D.H.)	1.0g
NH ₄ NO ₃	1.0g
MgSO ₄ .7H ₂ O	0.1g
Ca(NO ₃) ₂ .4H ₂ O	0.05g
Glucose	1.0g
K ₂ HPO ₄	1.0g
KH ₂ PO ₄	1.0g
Distilled H ₂ O to 1 litre.	
Final pH adjusted to 6.8	

Low Salt Broth (L.S.B.) (Barnet and Vincent, 1971)

Yeast Extract (Difco)	0.5g
Sucrose	2.5g
Distilled H ₂ O to 1 litre	
Final pH adjusted to 6.8	

Nutrient Agar (N.A.)

Oxoid nutrient agar was prepared, from dehydrated stock, according to the manufacturers instructions.

Rhizobium Defined Medium (R.D.M.) (Brown and Dilworth, 1975)

Glucose	2.5g
KH_2PO_4	1.4g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.25g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.02g
NaCl	0.2g
FeCl_3	6.6mg
E.D.T.A.	15.0mg
NH_4Cl	0.11g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.16mg
Na_2MoO_4	0.2mg
H_3BO_3	0.25mg
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.02mg
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	1.00 g
Thiamine Hydrochloride	1.00mg
Calcium Pantothenate	2.00mg
Biotin	1.00 g
Distilled H_2O to 1 litre.	
Final pH adjusted to 6.8	

Rhizobium Defined Agar (R.D.A.)

This was R.D.M. supplemented with 16g. Difco "bacto" agar per litre.

Glucose Minimal Agar (G.M.A.)

This was R.D.M. supplemented with 16g. Difco "noble" agar per litre.

ANTIBIOTIC PREPARATIONS

Where antibiotics were incorporated into media the following methods were used. Tetracycline hydrochloride and rifampicin were dissolved in methanol and added, without sterilization, to media to final concentrations of 10 μ g/ml. and 100 μ g/ml. respectively. Kanamycin sulphate and streptomycin sulphate were dissolved in distilled water, sterilized by membrane filtration, and added to media to a final concentration of 200 μ g/ml.

SOLUTIONS AND NODULATION TEST MEDIA

Phage Buffer (Clowes and Hayes, 1968)

Na ₂ HPO ₄	7.0g
KH ₂ PO ₄	3.0g
NaCl	4.0g
MgSO ₄ ·7H ₂ O	0.2g
Distilled H ₂ O to 1 litre.	
Final pH adjusted to 7.1	

Seedling Solution (Thornton, 1930)

Ca ₃ (PO ₄) ₂	2.0g
K ₂ HPO ₄	0.5g
MgSO ₄ ·7H ₂ O	0.2g
NaCl	0.1g
FePO ₄	1.0g
FeCl ₃	0.01g
Trace Element Solution	1ml.
Distilled H ₂ O to 1 litre	

Trace Element Solution

Bo	0.05%
Mn	0.05%
Zn	0.005%
Mo	0.005%

Cu 0.002%

Final pH adjusted to 6.5

Seedling Agar

Seedling agar was 15g of Difco "bacto" agar dissolved in 1 litre of seedling solution.

Acidified Mercuric Chloride Solution

HgCl₂ 0.2%
Concentrated HCl 5ml/litre

Water Agar

Difco Bacto Agar 16g
Distilled H₂O to 1 litre
Final pH adjusted to 6.8

Isolation of G-Series Strains

Clover plants were dug out of the ground from several sites around the Warwick University campus. The plant roots were cleansed of superficial soil and then washed thoroughly with running tap water. Only one nodule was taken from each plant root system. Before sterilisation a short piece of root tissue was left attached to the nodule to prevent diffusion of HgCl₂ into the centre of the nodule. Washed nodules were rinsed in 95% ethanol then immersed in acidified HgCl₂ for 3-4 minutes before washing in sterile, distilled water. To ensure that all HgCl₂ was removed the final washing step was repeated at least six times. To isolate the rhizobia from the sterilised nodule the nodule was aseptically transferred to a Y.M.A. plate, crushed with sterile forceps, and the milky fluid was streaked out for isolated colonies. Large, white, mucoid colonies arose after three to six days incubation at 30°. Only one colony was taken as a representative isolate from each nodule and each colony was restreaked at least three times from single colonies before it was considered

a pure culture. Twenty eight isolates were obtained by this method and were labelled G1 to G28.

The preliminary identification of these isolates as rhizobia was purely on the characteristics of slow growth rate and the production of extracellular slime on Y.M.A. plates. Any strains which were capable of growing at 37°, or on N.A. were discarded. In fact none of the strains which produced copious slime on Y.M.A. could grow on N.A., or at 37°. The efficiency of the surface sterilisation was demonstrated by comparing the growth from an uncrushed, unsterilised nodule with that obtained from a sterilised, uncrushed nodule. Surface sterilisation proved so effective that no growth at all appeared from the surface sterilised nodule until it was crushed open. All putative rhizobia were characterised further using antibiogram tests, rhizobiophage sensitivities and nodulation tests.

Nodulation Test Methods

Seed Coat Sterilisation

Legume seeds were surface sterilised using the same protocol as that used for nodule sterilisation. The plant cultivar used was Trifolium pratense var. Attasvede.

Germination of Surface Sterilised Seeds

Surface sterilised seeds were germinated for three days on water agar plates. All germinations were under sterile conditions, at room temperature, and in the dark. When the seedlings' roots were about two centimetres long the seedlings were aseptically transferred to seedling agar slopes, contained in 150mm by 30mm tubes. Sterile seedling solution was added up to the first few millimetres of the emerging root and the roots were protected from the light by wrapping silver foil around the tube. Plants were allowed to grow in a plant growth room at 20° with a day length of 12 hours.

Nodulation Assay Conditions

After seven days growth the tubes containing seedlings were inoculated with the rhizobial culture to be tested. Nodules appeared after about two weeks. Control plants were not inoculated with rhizobial cultures. Nodules were never observed on control plants. Initially the plant tests were performed with the complete plant system within a sterile test-tube, to prevent contamination. However, later tests were performed, by Dr Clive Ronson, in which the leaf system was allowed to develop outside of the sterile tube. This method yielded healthier plants and therefore, better results than the initial method. Where necessary the rhizobia were reisolated from the nodules for characterisation. All isolates tested were nodulation positive although the efficiency of nodulation varied from isolate to isolate.

Culture Maintenance

All cultures were purified from single colony isolates at least three times before they were stored on Y.M.A. slopes. Subcultures were made approximately every six months, and always from single colonies.

Incubations

For rhizobia most incubations were at 28-30° as static cultures in G.S.Y.C. or L.S.B. E.coli and P.aeruginosa were incubated statically at 37° in G.S.Y.C..

Spectrophotometry

The optical density of cultures was measured in 1ml cuvettes, at 540nm, using a Cecil 202 spectrophotometer.

Bacteriophage Lysate Production

All stock lysates were prepared from single plaque isolates which had been grown on the same host at least three times before the stock lysate was prepared. Two methods were used to prepare the lysate.

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1) Mitomycin-C Induction (M.C.I.) of Lysogens

Mid log phase cultures were incubated in the presence of mitomycin-C, at a final concentration of 0.5 µg/ml, until lysis occurred. Cellular debris was removed by low speed centrifugation and the supernatant was stored over chloroform after dialysis against phage buffer.

2) Soft Agar Method

The top layers from plates showing confluent lysis were pooled and mixed with an equal volume of phage buffer or G.S.Y.C. broth in the presence of 5% V/V Chloroform. Efficient mixing was maintained, using a magnetic stirrer, for periods of up to 30 minutes. The agar, cell debris and chloroform were removed by low speed centrifugation and the supernatant stored over chloroform at 4°. This method tended to yield viscous lysates due to the slime produced by the rhizobial host. Lysates produced by either of these methods achieved titres of 10^7 to $>10^{10}$, depending on the phage used.

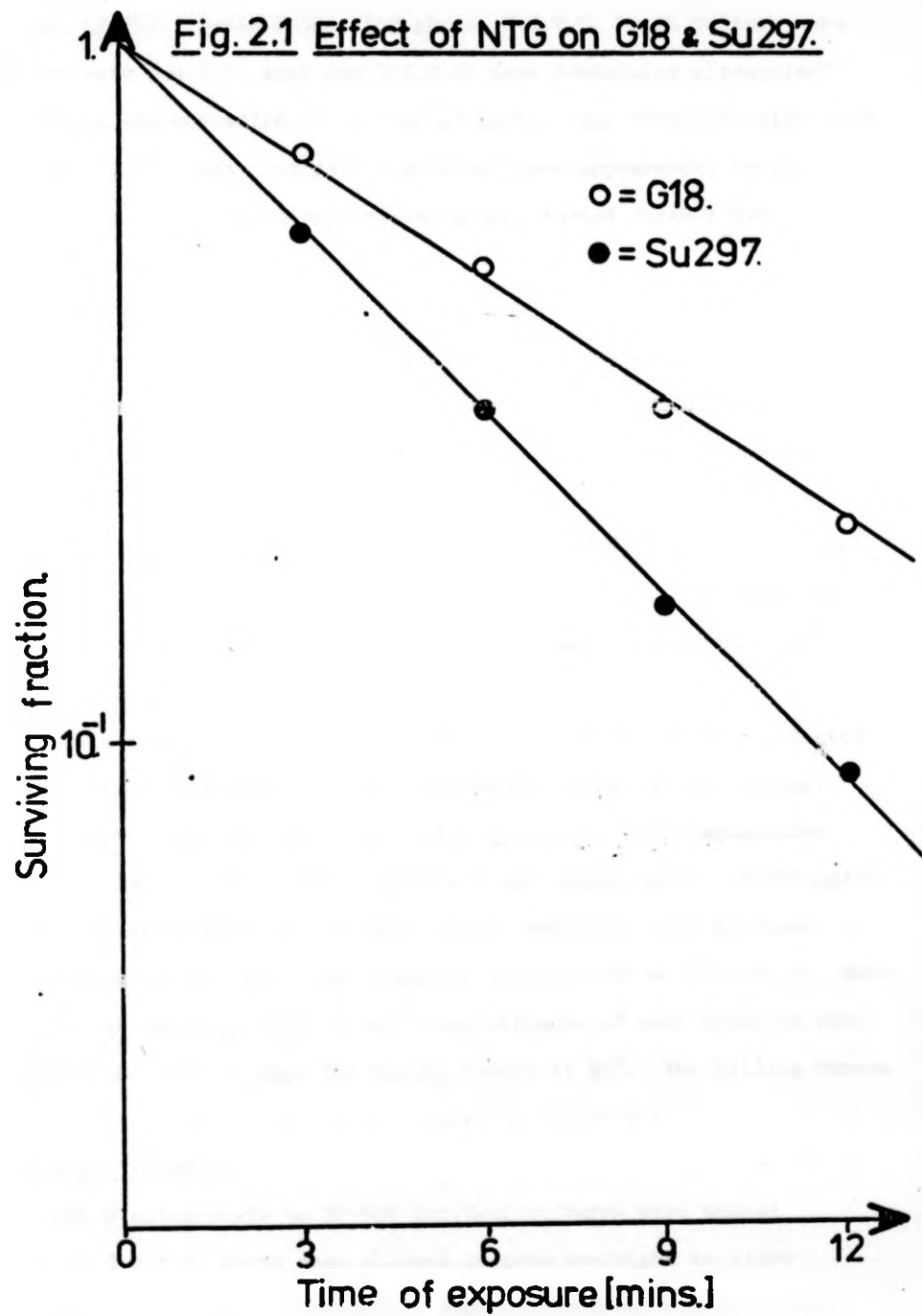
Plaque Assays

Phage lysates were diluted into G.S.Y.C. broth or phage buffer and 0.1ml samples of each dilution added to 0.2ml of a log phase rhizobial culture at an optical density of 0.2 to 0.3. Three millilitres of 0.6% top agar (G.S.Y.C.) were added to the phage/host mixture and this was poured over freshly poured G.S.Y.C. bottom agar and allowed to set. All plates were incubated at 28-30° for 2 days.

Phage/Bacteriocin Sensitivity Screens

Seeded top agar lawns were prepared from mid log phase cultures at an optical density of 0.2 to 0.3. One drop of the test lysate was spotted on to the set top agar and a control spot of sterile G.S.Y.C. was always added to the same plate as a control against dilution effects.

An alternative screening method for the identification of phage/bacteriocin producers was an adaptation of the method of Siddiqui



et. al. (1974). Cells from a log phase, G.S.Y.C. broth culture were patched onto G.S.Y.C. agar and G.S.Y.C. agar containing mitomycin-C at a final concentration of 0.2 to 1.0 $\mu\text{g/ml}$. Any colonies which grew well on G.S.Y.C. agar but gave a mottled/lysed appearance, or no growth at all, on the mitomycin plates were tested further for mitomycin sensitivity.

Antibiograms

All antibiograms were performed using Oxoid "Multodiscs". Various "Multodiscs" were used which had a variety of antibiotics impregnated into them. The antibiotics used; their codes and the concentrations in the discs are listed in Table 2.1. The "Multodiscs" were aseptically transferred onto the top of a G.S.Y.C. top agar layer, seeded with a log phase culture of the tester strain. The inhibition zones were measured in millimetres after 2 days incubation at 30°.

Killing Curves

Cells from log phase cultures of the tester strain were pelleted by low speed centrifugation then resuspended in 9ml of the culture supernatant. One millilitre of filter sterilised nitrosoguanidine (N.T.G.) (2mg/ml) was added to give a final concentration of 200 $\mu\text{g/ml}$. The pH was approximately 6.9 after N.T.G. addition. The cultures were shaken at 30° and 0.1ml aliquots were removed at intervals. Each aliquot was serially diluted and 0.1ml aliquots of each dilution were spread onto G.S.Y.C. agar for colony counts at 30°. The killing curves for the strains Su297 and Gl8 are shown in Figure 2.1.

Auxotroph Isolation

After mutagenesis to 30-50% survival cultures were washed twice in G.S.Y.C. broth then allowed to grow overnight to allow segregation of induced mutations. Serial dilutions of the culture were then plated out to isolated colonies on G.S.Y.C. agar. All colonies which arose were replicated onto G.M.A. and G.S.Y.C. agar to test for auxotrophy. Colonies which grew on G.S.Y.C. but not on

Table 2.1"Multodisc" antibiotics

Code	Antibiotic	Concentration (μ g)
Va.	Vancomycin	30.
PN.	Ampicillin	25.
Sxt.	Sulphamethoxazole/Trimethoprim	25.
Na.	Nalidixic Acid	30.
PB.	Polymixin B	300.
NV.	Novobiocin	5.
S.	Streptomycin	25.
SH.	Spectinomycin	25.
K.	Kanamycin	30.
N.	Neomycin	30.
E.	Erythromycin	50.
C.	Chloramphenicol	50.
PY.	Carbenicillin	100.
TE.	Tetracycline	50.
Rd.	Rifampicin	30.
Kf.	Cephalothin	25.
Ct.	Colistin Sulphate	10.
RL.	Sulphamethoxazole	25.

G.M.A. were selected as putative auxotrophs and further purified. This method generally yielded about 1% auxotrophs. The nutritional requirements for each auxotroph was determined by the pool method of Holliday (1956).

An alternative method of auxotroph selection was used. After mutagenesis and segregation, serial dilutions of the culture were plated out for isolated colonies on R.D.A.. The R.D.A. allowed very slight growth, of all auxotrophs, to occur. Thereafter any small colony which arose on the R.D.A. was tested for auxotrophy and nutritional requirements by the standard methods. This method was only used for strain G18 and it yielded 5% auxotrophs.

Bacterial and Phage Strains

The bacterial and phage strains used in this study are shown in Table 2.2.

Table 2.2. Bacterial and Phage strains

ORGANISM	STRAIN	SOURCE
<u>R.trifolii</u>	P2.	Dr. J. Lowe (University of Edinburgh)
	IDL.	
	W19	Dr. G. Atkins (Warwick University)
	NZP7	Dr. C. Ronson (Warwick University)
	"G-series"	This study
	"VW-series"	Mrs. Vicky Waddell (Warwick Univ.)
	Su297/F ₁ 32	Dr. Y. Barnet (University of New South Wales)
	Su298/533	
	Su297/31	
	NU18	
	RT 5.	Dr. E. Schwinghamer (Division of Plant Industry, G.S.I.R.O., Canberra)
	CC276.	
	TAl.	
	RO611	
	R52.	
<u>R.leguminosarum</u>	1001.	Rothamsted Experimental Station, Harpenden, Herts.
	1013.	
	1024.	
	1038.	
	2035.	
<u>R.meliloti</u>	WU60	Dr. M. Dilworth (Murdoch University)
<u>A.tumefaciens</u>		Dr. H. Dalton (Warwick University)
<u>E.coli.</u>	1230	Dr. J. Beringer (John Innes Inst.)
<u>P.aeruginosa</u>	PAT904REV1.	Dr. M. Day (U.W.I.S.T.)

Table 2.2 (Contd)

PHAGE	SOURCE
ØC, ØI, Øa.	Dr. G. Atkins
Ø7, Ø8, Øi.	This study.
ØPRR1.	Dr. M. Day (U.W.I.S.T.)

CHAPTER III

RESTRICTION AND MODIFICATION

AND

HOST RANGE STUDIES

Rhizobiophages were originally isolated in 1932 by Laird (Laird, 1932). In common with other bacteriophages the phages of Rhizobium can be classified into two broad groups; namely the temperate phages and the virulent phages. The virulent phages generally lyse the host cell which they infect. On the other hand, the temperate phages may develop in either of two ways once inside the host cell. Like the virulent phages the temperate phage may lyse the host or alternatively, the temperate phage nucleic acid may enter into a "molecular symbiosis" with the host such that the phage nucleic acid is propagated from one generation to another as the host cell divides. In such a situation the host cell is said to be lysogenic and it suffers no deleterious effects from the resident phage nucleic acid. In lysogeny the phage DNA may be physically integrated into the resident bacterial genome, as with λ in E.coli K12, or it may exist autonomously as an extrachromosomal replicon, like Pl_{lc} in E.coli (Hayes, 1968). Both virulent and temperate rhizobiophages have been isolated and described (Laird, 1932; Marshall, 1956; Ordogh and Szende, 1961; Barnet, 1972; Atkins, 1973). To date only phages carrying DNA have been isolated for Rhizobium. There have been no reports of single-stranded DNA or RNA-containing phages analogous to those found for E.coli. Neither have there been any reports on the derivation of strains lacking temperate phage attachment sites, analogous to the λ_{att}^A strains of E.coli.

An interesting feature of some bacterial strains is their ability to resist the lethal effects of virulent and temperate phage infections by producing enzymes which degrade the nucleic acid of the infecting agent. This phenomenon is called "restriction" and, correspondingly, the enzymes elaborated under these conditions are called restriction endonucleases (for review see Arber, 1974). The genetic capability to produce these enzymes can be conferred upon the host cell by virtue

of its own genome (Arber, 1968); the genes of some plasmids (Arber and Wauters-Willems, 1970); or even the genes of some phages (Arber and Dussoix, 1962).

Obviously the host cell has to have a mechanism whereby it renders its own DNA insensitive to restriction, otherwise the possession of such endonucleases would be a lethal event. The cell escapes restriction of its own DNA by methylating specific bases at particular positions along the genome thereby physically preventing access of the endonuclease to its own specific sites. This phenomenon is called "modification", and a variety of phages and episomes, as well as the host chromosome, are substrates for this methylation (Arber, 1968; Glover and Colson, 1969). Other phages are protected from restriction by their pattern of glucosylation rather than methylation. The T-even phages, T2 and T₄, are examples of this class of phages which are protected by glucosylation at their hydroxymethyl-cytosine residues. When such phages are grown on hosts which are incapable of glucosylating the phage DNA they become sensitive to host nucleases (Revel and Luria, 1970). Correspondingly when the phages which are subject to modifying methylation are grown in hosts in the absence of methionine the phage DNA is not modified to the new host specificity (Arber, 1965). Interestingly, only one strand of the DNA needs to be modified to prevent restriction (Arber and Dussoix, 1962), thus protecting newly replicated DNA.

Because restriction and modification are under genetic control it has proved possible to isolate strains defective in either or both systems. However, although it is possible to isolate modification-less mutants, by definition, they cannot be isolated in restriction - positive strains because such a condition would be lethal. In fact there are three genes involved in the restriction and modification system of E.coli. Apart from the hcr and hcm genes which code for the

restriction nuclease and modification methylase respectively, there is another gene, has, which controls the synthesis of a polypeptide involved in the recognition of the DNA sites which are to be restricted or modified. The various mutational types have been reviewed by Lewin (1974).

To date, a variety of restriction enzymes have been purified from several bacterial genera and their modes of action have been intensively studied. The enzymes have been classified into two types according to their obligate cofactor requirements. The type I enzymes depend on ATP, S-adenosyl methionine and magnesium ions for their activity whereas the type II enzymes only require magnesium ions (Marx, 1973). Of the two classes of enzyme, the type II enzymes are smaller in subunit composition and more specific in their cleavage sites. Some of the type II enzymes recognise palindromic base sequences and produce "staggered nicks" in the recognised sequence of the genome thereby exposing cohesive ends. DNA from various sources can be cleaved in such a fashion and ligated into vector plasmids (Morrow *et. al.*, 1974) or phages (Murray *et. al.*, 1977), using DNA ligase to link the cohesive ends of the heterologous DNA species. Such novel recombinant DNA molecules have a variety of uses (Primrose, 1977) although to date there are only three reports of the *in vitro* construction of recombinant molecules containing rhizobial DNA (Jacob *et. al.*, 1976; Neve *et. al.*, 1979; Julliot and Boistard, 1979).

There have been reports on restriction and modification (R & M) in Rhizobium (Schwinghamer, 1965, 1966; Barnet, 1969; Schwinghamer, 1971). For one strain of R. leguminosarum the restriction of rhizobiophage could be prevented by sub-lethal heating or U.V.-irradiation of the bacterial host. Alternatively the restriction could be overcome by infecting the host at high multiplicities (Schwinghamer, 1966). Such treatments have previously been shown to inactivate or avoid the restriction system of other genera (Uetake, 1964; Holloway, 1965).

There have been no reports of the isolation and characterisation of any rhizobial restriction enzymes although it has been suggested that the phenomenon of restriction may be widely distributed among the rhizobia (Schwinghamer, 1965). This makes rhizobial restriction worthy of study because R & M have a genetic basis and are therefore pertinent to the overall study of rhizobial genetics. Also, restriction can act as a significant barrier to genetic exchange and so its presence or absence must be determined in genetical experiments. Finally, if restriction is indeed a prominent feature in the rhizobia then the restriction enzymes can be isolated and may be of future use in in-vitro genetic manipulations.

The conventional method used to screen bacterial strains for restriction involved the use of phages. Bacteria which can restrict foreign DNA are detected as strains which only allow low efficiencies of plating by any particular phage. Such putative restricting hosts can then be investigated more thoroughly. It is helpful then to have a phage which can be grown to high titre and which forms large clear plaques on a variety of hosts. Rhizobiophage, ϕC , is such a phage, and it also has the advantage that it has a defined genetical system (Atkins, 1973b) and has been characterised biophysically (Atkins, 1973a; Atkins and Avery, 1974). An alternative screening method for the detection of restricting hosts has been developed in which λ DNA is incubated, with extracts of the tester strain, and then examined for degradation products (Roberts, 1976).

MATERIALS AND METHODS

First screen for ϕ C-sensitive strains

A lysate of ϕ C was prepared on W19 to a titre of $\sim 10^9$ p.f.u./ml.. This phage (designated ϕ C.W19) was used in crude cross-streak tests against colonies of a variety of rhizobial strains, to test for ϕ C-sensitivity. All cross-streaks were on R.D.A. plates and all incubations were at 30°C. Any strain showing lysis at the phage/host intersection was regarded as a ϕ C-sensitive isolate. This ϕ C-sensitivity was subsequently checked in standard spot tests before the strains were used in restriction tests.

Generation of Lysates for restriction assays

All lysates were prepared from single plaque isolates and had been grown on the relevant host for at least three cycles of growth before stock lysates were used for the restriction assays.

RESULTS AND DISCUSSION

Cross-streaks for ϕ C-sensitive hosts

The results were extremely difficult to read due to the copious slime produced by rhizobia on RDA. Even those recorded as positive were not clearly lysed at the phage/host streak intersection. From the results, presented in Table 3.1, it can be seen that G18 gave a positive result. This is surprising because subsequent tests showed that G18 only plates ϕ C with low efficiency. Of 32 strains tested only 9, or 28%, were ϕ C-sensitive by this test. However subsequent ϕ C sensitivity screens using standard spot tests produced a higher incidence of ϕ C-sensitivity among a variety of rhizobia (See Table 3.12). Consequently these cross-streak tests are probably not an accurate method of assessing ϕ C-sensitivity.

The nine putative ϕ C-sensitive strains all proved ϕ C-sensitive by the spot test method. On titration however, there was great variation in the size and uniformity of plaque morphology between strains. In the case of G28 the plaques formed were so small that they became difficult to work with and so the analysis for R & M was only conducted on the remaining eight ϕ C-sensitive strains. The results of the reciprocal testing of all lysates are presented in Tables 3.2 to 3.9 with all the data summarised in Table 3.10.

Restriction Assays

There are several points to be borne in mind when considering these data. There was a pronounced variation in plaque morphology which seemed to be critically dependent on the physiological state of the recipient rhizobia. This dependence of plaque forming ability on the physiology of the host was far more acute than in other genera such as E.coli (S. Primrose - pers. comm.). Also, with so many lysates to produce, and with so many plaque assays to perform, variability in

Table 3.1Results of cross-streaks for OC-sensitivity

STRAIN	RESULT	STRAIN	RESULT
G1	R	G17	S
G2	R	G18	S
G3	R	G19	R
G4	R	G20	R
G5	R	G21	R
G6	R	G22	R
G7	R	G23	R
G8	R	G24	R
G9	R	G25	R
G10	R	G26	R
G11	R	G27	R
G12	R	G28	R
G13	S	W19	S
G14	S	P2	S
G15	S	IDL	S
G16	R	Wu60	R

R = Resistant

S = Sensitive

apparent titre and plaque heterogeneity was common. Overall, a variation in titre of ± 1 to $1\frac{1}{2}$ logs could be ascribed to simple irreproducibility in these experiments purely due to the vagaries of the plaque forming system.

The data in Table 3.10 shows that the titre of ϕ C on any host was affected by the host on which the phage was previously grown. ϕ C plates more efficiently on W19 and G15 than on any other strain irrespective of the previous host e.g., even when ϕ C was grown on IDL the phage plated about two logs more efficiently on W19 and G18 than it did on IDL itself. This effect is a common feature throughout this study although the reasons for it are unknown. Most likely the effect is due to different rates of adsorption of the phage to the cell surface. If ϕ C adsorbed more efficiently to W19 and G15 than the other hosts then the apparent e.o.p. of ϕ C on these two hosts would always be as high, or higher, than the e.o.p. on the other hosts. Of course, this would only apply if no pronounced restriction of ϕ C, by W19 and G15, occurred.

If a variation of $\pm 1\frac{1}{2}$ logs is allowed in the analysis then there are no obvious putative restricting hosts in this group, except G18. It is possible that every other variation could be attributable to general fluctuations in the experiments and effects of adsorption efficiencies. However the variations in e.o.p. are so small relative to the natural background variation that further study on hosts other than G18 would have proved difficult. Because the effect on G18 was so dramatic then this was the strain chosen for further study. If G18 is a restricting host, then by necessity, it must also be a modifying host. Under such conditions all of the other hosts would have to be restrictionless. This is because, within the broad limits of the experimental system, passage of ϕ C through G18 does not radically reduce the e.o.p. of ϕ C.G18 on the other hosts. So, does G18 restrict ϕ C DNA? If it does then ϕ C.W19.G18 would form plaques as efficiently on G18 as on W19, unless there is also some adsorption phenomenon acting in conjunction with R & M. As can be seen

Table 3.2Titration of OC.W19 on various hosts

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	5x10 ⁹	1
P2	1x10 ⁸	2x10 ⁻²
IDL	2x10 ⁸	4x10 ⁻²
G13	5x10 ⁶	1x10 ⁻³
G14	1x10 ⁹	2x10 ⁻¹
G15	5x10 ⁹	1
G17	1x10 ⁹	2x10 ⁻¹
G18	4x10 ³	8x10 ⁻⁷

All titres are corrected to nearest whole number. Each final e.o.p. and p.f.u. is an average of at least two experiments and sometimes as many as six. This applies to all tabulated results in Tables 3.2 to 3.9.

Table 3.3Titration of ØC.P2 on various hosts

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	3x10 ⁹	3x10 ¹
P2	1x10 ⁸	1
IDL	1x10 ⁶	1x10 ⁻²
G13	1x10 ⁶	1x10 ⁻²
G14	6x10 ⁸	6x10 ⁰
G15	8x10 ⁷	8x10 ⁻¹
G17	1x10 ⁷	1x10 ⁻¹
G18	1x10 ²	1x10 ⁻⁶

Table 3.4Titration of ØC.IDL on various hosts

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	4x10 ⁸	2x10 ²
P2	6x10 ⁷	3x10 ¹
IDL	2x10 ⁶	1
G13	2x10 ⁵	1x10 ⁻¹
G14	2x10 ⁸	1x10 ²
G15	2x10 ⁸	1x10 ²
G17	8x10 ⁶	4x10 ⁰
G18	4x10 ¹	2x10 ⁻⁵

Table 3.5 Titration of ØC.G13 on various hosts

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	6x10 ⁹	2x10 ³
P2	9x10 ⁸	3x10 ²
IDL	6x10 ⁷	2x10 ¹
G13	3x10 ⁶	1
G14	3x10 ⁹	1x10 ³
G15	5x10 ⁹	2x10 ³
G17	8x10 ⁸	3x10 ²
G18	1x10 ⁴	3x10 ⁻³

Table 3.6Titration of ØC.G14 on various hosts

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	2x10 ⁸	7x10 ⁰
P2	3x10 ⁶	1x10 ⁻¹
IDL	9x10 ⁴	3x10 ⁻³
G13	9x10 ²	3x10 ⁻⁵
G14	3x10 ⁷	1
G15	1x10 ⁸	3x10 ⁰
G17	2x10 ⁶	7x10 ⁻²
G18	6x10 ¹	2x10 ⁻⁶

Table 3.7Titration of ØC.G15 on various hosts

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	1x10 ⁸	1x10 ⁰
P2	5x10 ⁶	5x10 ⁻²
IDL	2x10 ⁶	2x10 ⁻²
G13	9x10 ⁴	9x10 ⁻⁴
G14	2x10 ⁷	2x10 ⁻¹
G15	1x10 ⁸	1
G17	4x10 ⁶	4x10 ⁻²
G18	5x10 ¹	5x10 ⁻⁷

Table 3.8Titration of PC.G17 on various hosts

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	7x10 ⁹	1x10 ¹
P2	5x10 ⁷	7x10 ⁻²
IDL	8x10 ⁷	1x10 ⁻¹
G13	2x10 ⁶	3x10 ⁻³
G14	2x10 ⁹	3x10 ⁰
G15	9x10 ⁸	1x10 ¹
G17	7x10 ⁸	1
G18	1x10 ⁴	1x10 ⁻⁵

Table 3.9 Titration of OC.G18 on various hosts

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	1x10 ⁷	1x10 ¹
P2	5x10 ⁵	6x10 ⁻¹
IDL	3x10 ⁵	3x10 ⁻¹
G13	4x10 ³	4x10 ⁻³
G14	6x10 ⁵	7x10 ⁻¹
G15	9x10 ⁶	1x10 ¹
G17	2x10 ⁵	2x10 ⁻¹
G18	9x10 ⁵	1

Table 3.10 Efficiency of plating of ϕC on various hosts
(summary of e.o.p. in tables 3.2 to 3.9)

$\frac{x}{y}$	W19	P2	IDL	G13	G14	G15	G17	G18
W19	1	3×10^{-1}	2×10^2	2×10^3	7×10^0	1×10^0	1×10^{-1}	1×10^{-1}
P2	2×10^{-2}	1	3×10^{-1}	3×10^2	1×10^{-1}	5×10^{-2}	7×10^{-2}	6×10^{-1}
IDL	4×10^{-2}	1×10^{-2}	1	2×10^1	3×10^{-3}	2×10^{-2}	1×10^{-1}	3×10^{-1}
G13	1×10^{-3}	1×10^{-2}	1×10^{-1}	1	3×10^{-5}	9×10^{-4}	3×10^{-3}	4×10^{-3}
G14	2×10^{-1}	6×10^0	1×10^2	1×10^3	1	2×10^{-1}	3×10^0	7×10^{-1}
G15	1×10^0	8×10^{-1}	1×10^2	2×10^3	3×10^0	1	1×10^{-1}	1×10^{-1}
G17	2×10^{-1}	1×10^{-1}	4×10^0	3×10^2	7×10^{-2}	4×10^{-2}	1	2×10^{-1}
G18	8×10^{-7}	1×10^{-6}	2×10^{-5}	3×10^{-3}	2×10^{-6}	5×10^{-7}	1×10^{-5}	1

x = last host for ϕC
y = new host strain

from the results in Table 3.10, ϕ C.W19.G18 plated less well, or as well, on G18 as on W19, but certainly no better. The progeny phage from this passage through W19 would be expected to plate less efficiently on G18 than on W19, if G18 is restricting and modifying ϕ C DNA. The relative e.o.p. would be expected to be about 10^{-6} from previous data.

From the results in Table 3.11 it can be seen that no reduction in the e.o.p. took place in such experiments. Therefore, once ϕ C had been grown on G18 the progeny phage seemed to be altered in such a way that they plated on W19 and G18 with approximately the same efficiency. Passage of such progeny phage through W19 again did not appear to radically affect this ability to form plaques on either host, although a slight adsorption effect may have been present.

So rather than these results suggesting phage restriction it appears that a more likely explanation is that passage through G18 selects host range mutants of ϕ C. A similar effect occurs between G18 and all other strains tested here. However the contributory effects of what are likely to be adsorption differences have a more pronounced bearing on the e.o.p.'s than in the G18-W19/G15 system. Therefore, contrary to earlier expectation, G18 is not a restricting host for ϕ C but host range mutants of ϕ C can be selected on G18 at a variety of frequencies dependent on the host on which ϕ C was previously grown.

At least for this system no definite R & M appears to exist. ϕ C is a very large phage with a molecular weight of $\sim 2 \times 10^8$ (Atkins and Avery, 1974). Because it is thought that the extent of restriction of DNA is greater with increasing molecular weight (Arber and Linn, 1969; Roulland-Dussoix and Boyer, 1969) then it does seem surprising that no obvious R & M of ϕ C occurs in a group of strains from a genus which, Schwinghamer suggested, might exhibit extensive R & M properties (see discussion of Schwinghamer, 1965). However, this observation, albeit preliminary, is in agreement with the observations of other workers regarding the relatively

Table 3.11 Titration of ØC.W19.G18.W19 on W19 and G18

HOST	p.f.u.ml ⁻¹	e.o.p.
A)		
W19	3.8×10^7	1
G18	2.2×10^7	0.58
B)		
W19	2.5×10^7	1
G18	9.5×10^6	0.38
C)		
W19	4.2×10^7	1
G18	7×10^6	0.6

A = ØC.W19.G18.W19¹

B = ØC.W19.G18.W19²

C = ØC.W19.G18.W19³

high levels of intra and interspecific genetic exchange among the rhizobia (Johnston and Beringer, 1977). If R & M were widely distributed one would not expect to have high efficiency transfer of DNA in such interspecific matings. Consequently R & M may not be as widely distributed among the rhizobia as Schwinghamer originally supposed.

Another alternative is that ϕ C has a genetic capability analogous to that conferred on the coliphage T7 by the O.3 gene. The O.3 gene in T7 codes for an ability to antagonise the effects of host restriction (Eskin *et. al.*, 1973; Studier, 1975). If ϕ C possessed such a genetic capability no restriction of ϕ C DNA would be evident at all in vivo although degradation would be productive in vitro when the phage DNA was incubated with host cell extracts containing the proposed restriction enzyme(s) (Eskin *et. al.*, 1973).

Rhizobiophage Host Range Studies

Because no restricting hosts for ϕ C were found in the first screen, a variety of other rhizobia were screened for ϕ C sensitivity in an attempt to identify any potential restricting hosts. These rhizobial strains were also tested for sensitivity to the unrelated phages ϕ 7, ϕ i, ϕ 8 and ϕ 7^c, which are supposedly subject to restriction by an R.trifolii host (Barnet, 1969). When the ϕ C-sensitive isolates were identified they were spot tested against serial dilutions of ϕ C.W19 to screen for potential restriction. Such ϕ C-sensitive hosts were also spot tested against the ϕ C-related phages ϕ I and ϕ a (Atkins, 1973; Atkins and Avery, 1974) to see if the host ranges were similar.

The results of the phage sensitivity spot tests are presented in Table 3.12. Of all the strains tested 67% proved sensitive to ϕ C.W19. Hence ϕ C must be particularly catholic in its host range. This result is in stark contrast to phages ϕ i, ϕ 7, and ϕ 8 which appear to have a very restricted host range. If the mode of infection of each strain by ϕ C is the same then there must be some common phage receptor site in all susceptible strains. At this stage it can be seen that ϕ C can infect

Table 3.12

Phage Sensitivity Spot Test Results

STRAIN	Ø7.Nu18	Øi.Nu18	Ø8.Nu18	Ø7 ^c .297	ØC.W19
VW1	-	-	-	-	-
VW2	-	-	-	-	S
VW3	-	-	-	-	-
VW5	-	-	-	-	S
VW6	-	-	-	-	-
VW8	-	-	-	-	S
VW9	-	-	-	-	S
VW10	-	-	-	-	S
VW11	-	-	-	-	S
VW12	-	-	-	-	-
VW13	-	-	-	-	S
VW14	-	-	-	-	-
VW15	-	-	-	-	-
VW16	-	-	-	-	S
VW18	-	-	-	-	-
VW19	-	-	-	-	S
VW20	-	-	-	-	S
VW21	-	-	-	-	S
VW22	-	-	-	-	S
VW23	-	-	-	-	S
VW24	-	-	-	-	-
VW25	-	-	-	-	S
VW27	-	-	-	-	S
VW28	-	-	-	-	S
VW29	-	-	-	-	S
VW31	-	-	-	-	S
VW32	-	-	-	-	-
VW33	-	-	-	-	-
VW34	-	-	-	-	S
VW36	-	-	-	-	S
VW38/1	-	-	-	-	S
VW38/2	-	-	-	-	S
VW39	-	-	-	-	S
VW40	-	-	-	-	-
VW51	-	-	-	-	-

Table 3.12 (contd)

STRAIN	Ø7.Nul8	Øi.Nul8	Ø8.Nul8	Ø7 ^c .297	ØC.W19
VW53	-	-	-	-	S
VW54	-	-	-	-	-
VW56	-	-	-	-	S
VW57	-	-	-	-	-
VW58	-	-	-	-	S
VW59	-	-	-	-	S
VW60	-	-	-	-	S
VW61	-	-	-	-	S
VW62	-	-	-	-	S
VW63	-	-	-	-	S
VW64	-	-	-	-	-
VW65	-	-	-	-	S
VW66	-	-	-	-	-
VW67	-	-	-	-	S
VW68	-	-	-	-	-
TA1	-	-	-	-	S
R0611	-	-	-	-	S
R52	-	-	-	-	S
CC276	-	-	-	-	S
W19	-	-	-	-	S
Nul8	S	S	S	S	-
Wu60	-	-	-	-	-
Agrobacterium	-	-	-	-	-
2035	-	-	-	-	-
1001	-	-	-	-	S
1038	-	-	-	-	S
1013	-	-	-	-	S
1024	-	-	-	-	S

S = sensitive

- = resistant

Titres :- Ø7.Nul8 ; 10^8 pfu/ml.
 Øi.Nul8 ; 10^8 pfu/ml.
 Ø8.Nul8 ; 5×10^8 pfu/ml.
 Ø7^c.297 ; 3×10^9 pfu/ml.
 ØC.W19 ; 3×10^9 pfu/ml.

R.trifolii and R.leguminosarum but neither of the R.meliloti nor Agrobacterium strains tested. The inability to lyse the latter two strains may be insignificant but the fact that ØC has an "interspecies specificity" is in agreement with other data which suggests that R.trifolii and R.leguminosarum are very closely related in genetical (Johnston and Beringer, 1977) and numerical taxonomic terms (Graham, 1964).

The results of the lysate dilution spot test assays on the ØC - sensitive strains are presented in Table 3.13. An analysis of the data reveals the following salient points. All ØC-sensitive strains are ØI sensitive although the relative sensitivity of any strain to ØC or to ØI may vary i.e. a strain may be more or less sensitive to ØC than it is to ØI. This can only be inferred from the intensity of the spot test reactions and not from actual e.o.p.'s. Some strains are more sensitive, to both phages, than other strains e.g., VW28 and VW10 are more sensitive, to ØC, than VW11 or VW21. Although all of the observations pertaining to these spot tests are not recorded in the table there was a great variation in the "quality" of the plaques, where isolated plaques were noted. The size of plaques varied from clear pin-point to very large plaques up to 5mm diameter. There were several potential restricting hosts which reacted to ØC in a manner analogous to Gl8's reaction e.g., VW15, VW11, VW21, VW25, VW29, VW34, VW36, VW52, VW53, VW55, VW59, VW61, VW65, CC276. This represents 14/38 or ~36% of the ØC sensitive strains which show some reduction in the e.o.p. of ØC.W19 relative to the value for W19 itself. Certain strains showed a very slight turbidity when challenged by Øa.W19; ØC.W19 and ØI.W19. This effect may be due to capsule depolymerisation caused by endoglycosidase activity free in the phage lysate or as some structural component of the phage particles themselves (Sutherland, 1967; Stirn, 1968; Stirn and Freund-Molbert, 1971; Stirn *et. al.*, 1971; Besaler *et. al.*, 1975; Eichholtz *et. al.*, 1975; Rieger *et. al.*, 1975). The same activity is probably responsible for the

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Table 3.13

Lysate dilution spot test assays

HOST	$\phi C 10^{-2}$	$\phi C 10^{-4}$	$\phi C 10^{-6}$	approx p.f.u.	ϕI	ϕA
VW2	C.L.	C.L.	I.P.	10^8	C.L.	-
VW5	T.L.	-	-	10^5	T.L.	-
VW8	C.L.	C.L.	I.P.	10^8	C.L.	-
VW9	C.L.	C.L.	I.P.	10^8	C.L.	I.P.
VW10	C.L.	C.L.	I.P.	10^8	C.L.	-
VW11	T.L.	-	-	10^5	C.L.	T.L.
VW13	C.L.	C.L.	I.P.	10^8	C.L.	-
VW16	C.L.	C.L.	I.P.	10^8	T.L.	I.P.
VW19	C.L.	I.P.	-	10^6	C.L.	-
VW20	C.L.	C.L.	I.P.	10^8	C.L.	I.P.
VW21	T.L.	-	-	10^5	C.L.	-
VW22	C.L.	C.L.	I.P.	10^8	C.L.	I.P.
VW23	C.L.	C.L.	I.P.	10^8	C.L.	I.P.
VW25	T.L.	-	-	10^5	C.L.	T.L.
VW28	T.L.	-	-	10^5	C.L.	-
VW29	T.L.	-	-	10^5	C.L.	I.P.
VW31	C.L.	C.L.	I.P.	10^8	C.L.	I.P.
VW34	T.L.	-	-	10^5	T.L.	T.L.
VW36	T.L.	-	-	10^5	T.L.	T.L.
VW38/1	C.L.	C.L.	I.P.	10^8	C.L.	I.P.
VW38/2	C.L.	C.L.	I.P.	10^8	C.L.	I.P.
VW39	C.L.	C.L.	I.P.	10^8	C.L.	T.L.
VW52	T.L.	-	-	10^5	T.L.	T.L.
VW53	T.L.	-	-	10^5	T.L.	T.L.
VW55	C.L.	-	-	10^5	T.L.	-
VW56	C.L.	C.L.	I.P.	10^8	C.L.	-

Table 3.13 (contd)

HOST	$\phi C 10^{-2}$	$\phi C 10^{-4}$	$\phi C 10^{-6}$	approx p.f.u.	ϕI	ϕA
VW58	C.L.	C.L.	I.P.	10^8	C.L.	I.P.
VW59	T.L.	-	-	10^5	T.L.	I.P.
VW60	C.L.	C.L.	I.P.	10^8	C.L.	-
VW61	C.L.	C.L.	I.P.	10^8	C.L.	I.P.
VW62	C.L.	C.L.	I.P.	10^8	C.L.	-
VW63	C.L.	C.L.	I.P.	10^8	C.L.	-
VW65	T.L.	-	-	10^5	C.L.	-
VW67	C.L.	C.L.	I.P.	10^8	T.L.	-
W19	C.L.	C.L.	I.P.	10^8	C.L.	I.P.
NZP7	C.L.	C.L.	I.P.	10^8	C.L.	-
CC276	T.L.	-	-	10^5	T.L.	-
TA1	C.L.	C.L.	I.P.	10^8	C.L.	-
RO611	C.L.	C.L.	I.P.	10^8	C.L.	-
R52	C.L.	C.L.	I.P.	10^8	C.L.	-
G18	T.L.	-	-	10^5	T.L.	-

Titres:- $\phi C(NEAT) 2 \times 10^9$ pfu/ml.
 $\phi I(NEAT) 5 \times 10^7$ pfu/ml.
 $\phi A(NEAT) 1 \times 10^3$ pfu/ml.

C.L. = clear lysis
T.L. = turbid lysis
I.P. = isolated plaques

genesis of the halo around the ØC, ØI and Øa plaques, because when plates exhibiting such halo effects are left several days after all bacterial lawn growth has ceased, the halo effect gradually extends across the complete lawn.

Of the strains which were potential restriction positive hosts for ØC, two were chosen for further study; namely VW52 and VW53. In an identical fashion to the analysis of e.o.p. on G18, ØC was grown on both strains and titred on W19. The results are presented in Table 3.14. The data reveal an identical result to those results obtained in the G18 analysis. The reduction in e.o.p. of ØC.W19 on VW52 and VW53 is again due to the selection of host range mutants. Once again there is no evidence from these results which suggests that R & M operate in the rhizobial isolates examined here. Perhaps R & M may be the cause of the reduced e.o.p. in other strains but it seems equally likely that their phenotypic "restriction" of ØC could be due to poor phage adsorption and, therefore, host range mutant selection.

As mentioned in the introduction restriction can act as a barrier to genetic exchange. Since there was published evidence in favour of the idea that restriction was widely spread among the rhizobia (Schwinghamer, 1965) this study was undertaken to ascertain just how frequently R & M did occur in this genus. The results from this study are not in agreement with Schwinghamer's suggestion. It is interesting, however, that "phenotypic restriction" is not always due to the classical effects of restriction endonucleases. Kruger et.al. (1977) have shown that, when passaging phage T7 and T3 derivatives between E.coli strains, a phenotypic host-controlled restriction and modification occurred. However, this phenomenon of phenotypic restriction and modification was not due to "classical" R & M of phage DNA but depended on reversible alteration of the absorption capacity of the phages, and was host determined. Hence Kruger et.al. (1977) suggest that all recorded cases of phenotypic R & M need not be due to

Table 3.14

Titration of ØC.W19

(a)

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	2×10^8	1
VW52	4.4×10^2	2.2×10^{-6}
VW53	6.2×10^2	3.1×10^{-6}

Titration of ØC.W19.VW52

(b)

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	7.6×10^8	1.4×10^1
VW52	5.4×10^7	1

Titration of ØC.W19.VW53

(c)

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	2.2×10^8	7×10^0
VW53	3.1×10^7	1

Titration of ØC.W19.VW52.W19

(d)

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	5.0×10^6	1
VW52	1.0×10^6	0.20

Titration of ØC.W19.VW53.W19

(e)

STRAIN	p.f.u.ml ⁻¹	e.o.p.
W19	8.2×10^6	1
VW53	2.3×10^6	0.36

modification of phage DNA but may, in some cases, be due to this "non-classical" R & M. Since there is no published work, by Schwinghamer or others, which describes the isolation and characterisation of a rhizobial restriction enzyme then the possibility remains that the R & M system described by Schwinghamer (1965) could have been due to the adsorption effects of "non-classical" R & M as described by Kruger et.al. (1977). Hence, until a rhizobial restriction enzyme is isolated and characterised it cannot be said with certainty that the rhizobia possess a classical restriction and modification system.

CHAPTER IV

LYSOGENY AND BACTERIOCINOGENY

There are many reports which suggest that both lysogeny and bacteriocinogeny are widely distributed among the rhizobia (e.g., Marshall, 1956; Parker and Allen, 1957; Davies, 1958; Szende and Ordogh, 1960; Schwinghamer and Reinhardt, 1963; Kowalski, 1966; Roslycky, 1967; Schwinghamer and Belkengren, 1968; Schwinghamer, 1971; Lotz and Mayer, 1972; Schwinghamer et. al., 1973; Schwinghamer, 1975). Both phenomena are important for several reasons. Temperate phages and bacteriocins may be coded for by either chromosomal or extrachromosomal genetic elements and are therefore of considerable intrinsic genetic interest (Hayes, 1968; Reeves, 1972). Temperate phages may be useful as vectors of rhizobial DNA during transduction (Kowalski, 1971; Svab et. al., 1978). Also, plasmids which code for bacteriocins can be used as cloning vehicles for specific DNA sequences in genetic engineering experiments (Clarke and Carbon, 1975; Primrose, 1977). Finally bacteriocins and temperate phages may act as unwelcome contaminants in lysates and cultures used for transduction or conjugation studies. Because of these important features a simple screen for rhizobiocins and rhizobiophages was undertaken in an attempt to find out how widely distributed either phenomenon was in R.trifolii.

Methods

In the first screen the cultures to be tested were grown in G.S.Y.C. broth and lawns were made in top agar layers. Supernatants of each culture were spot tested on every lawn with the aid of a phage typing block. Some phage lysates were included as positive controls. In the second screen an adaptation of the rapid-plate method (Siddiqui et. al., 1974) was used. Strains known to be lysogenic were included as positive controls. In the third screen

the supernatants from a variety of rhizobial isolates were chloroform sterilised then tested against a small number of R.trifolii strains using the phage typing block replication method. In the fourth and final method a variety of chloroformed culture supernatants were tested against W19 by the standard top-agar spot test method.

Results and Discussion

From the results of the phage and bacteriocin screens in Table 4.1 there is no evidence for any phage or bacteriocin release from any of these isolates. All of the controls were positive even at high dilution. In the second screen, using the mitomycin C agar (MCA), the known lysogens reacted in the expected fashion by failing to grow in the presence of low mitomycin concentrations (Table 4.2). It is interesting that the "rough" mutants of G18 and G17, isolated as ϕ C-resistant mutants, were mitomycin sensitive whereas the mucoid G18 and G17 parents were resistant. This suggests that the uptake of mitomycin was more active in the rough strains than in the mucoid strains and this differential mitomycin sensitivity may be related to the quantity of extracellular slime produced by each strain. Although the rough strains had been isolated as ϕ C-resistant mutants they did not carry ϕ C, at least by the criterion of inability to release any phage into the supernatant. So, once again, there was no evidence for lysogeny or bacteriocinogeny in these tester strains.

In the third screen no obvious inhibitory effects were detected over and above those of the included controls (Table 4.3). The one exception to this observation was the apparent effect of VW28 supernatant on G18, where a definite lysis occurred. This was investigated further (see page 92). In the fourth screen the results were negative as in the previous three screens (Table 4.4). The spot test assays generally

Table 4.1 Spot tests for phage and bacteriocin release

RECIPIENT TESTER STRAIN	SUPERNATANT																PHAGE
G1	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	ØC
G3																	Ø1
G4																	ØA
G6																	
G7																	
G8																	
G9																	
G10																	
G12																	
G13																	
G14																	
G17																	
G18																	
G19																	
G20																	
G22																	
G23																	
G25																	
G26																	
G28																	
RT5																	
W19																	
Wu60																	
P2																	
IDL																	

NOT
TESTED

ALL -ve

CL CL CL
- - -
CL CL CL
CL CL CL

CL = clear lysis

yield completely unequivocal results and so the absence of detectable inhibitory effects on the growth of W19 lawns was very disappointing. It is interesting that no inhibition of W19 growth appears to take place in the presence of VW28 supernatant. This contrasts with the effect on G18, attributable to the same lysate, in the previous experiment.

These experiments yielded very little data on the distribution of lysogeny and bacteriocinogeny in the rhizobia. Neither do the results add credibility to the suggestions that both of these phenomena are widely distributed among the root-nodule bacteria (Ordogh and Szende, 1961; Schwinghamer, 1971). Overall the results from these experiments may be interpreted in several ways. Lysogeny and bacteriocinogeny may be rare phenomena in the sample of rhizobia examined here. Alternatively, lysogeny and bacteriocinogeny may indeed be prominent features in Rhizobium but the conditions under which phage and/or bacteriocins are released are somewhat exacting e.g., very high concentrations may be an obligate requirement for ~~detection~~ of activity by the methods used. Another possibility is that phage and bacteriocin release is common but no "indicator" strains are present in these tests i.e., all of the strains tested are resistant to all of the phage and bacteriocins present in the lysates. A similar situation has been shown to exist in the genus Streptobacterium (Stetter, 1977). In fact data from other workers (J Beringer, pers. comm.; S Primrose, pers. comm.) demonstrate that the conditions required for the detection of rhizobiophage and rhizobiocins may well be very exacting.

These screens were undertaken because published work suggested that bacteriocins and phages are widely distributed in Rhizobium (Ordogh and Szende, 1961; Schwinghamer, 1971). The data presented in this study contradicts these earlier conclusions. However, very recently, the results of this study have been contradicted by the discovery that bacteriocinogeny is very widely distributed in Rhizobium (Hirsch, 1979).

Table 4.2

Mitomycin sensitivity of various strains

STRAIN	($\mu\text{g} \cdot \text{ml}^{-1}$) MITOMYCIN CONCENTRATION				
	0	0.2	0.4	0.6	1.0
Su297/F ₁ 32	+	-	-	-	-
Su298/537	+	-	-	-	-
Su297(7)	+	-	-	-	-
Su297(7 ^{cr})	+	-	-	-	-
Su297/31	+	-	-	-	-
Nu18	+	+	+	+	+/-
W19	+	+	+	+	+/-
G15	+	+	+	+	+/-
P2	+	+	+	+	+/-
G13	+	+	+	+	+/-
IDL	+	+	+	+	+/-
G18	+	+	+	+	+/-
G18R1	+	+/-	-	-	-
G17	+	+	+	+	+/-
G14	+	+	+	+	+/-
N2P7	+	+	+	+	+/-
G17R1	+	+/-	-	-	-
G17R2	+	+/-	-	-	-
G1	+	+	+	+	+/-
G2	+	+	+	+	+/-
G3	+	+	+	+	+/-
G4	+	+	+	+	+/-
G5	+	+	+	+	+/-
G6	+	+	+	+	+/-
G7	+	+	+	+	+/-
G8	+	+	+	+	+/-
G9	+	+	+	+	+/-
G10	+	+	+	+	+/-
G11	+	+	+	+	+/-
G12	+	+	+	+	+/-
G16	+	+	+	+	+/-
G19	+	+	+	+	+/-
G20	+	+	+	+	+/-
G21	+	+	+	+	+/-
G22	+	+	+	+	+/-

+ = good growth +/- = weak growth
 - = no growth

Does VW28 release a phage or bacteriocin ?

As shown in Table 4.3, VW28 supernatant appears to have an inhibitory effect on G18. Originally it was suspected that this apparent inhibitor had really been ϕ C from a small well of lysate close to VW28 supernatant on the tester grid and so the assay was repeated in a more definitive spot test. When the filtered or chloroformed supernatants of 3-4 day old cultures of VW28 were spotted onto top agar lawns of G18 the results presented in Table 4.5 were obtained. G18 was inhibited by VW28 supernatant and a turbid lysis was noted compared with the very clear lysis due to ϕ C in the control spot. No lysis was detected in the dilution spot control. To determine whether or not this lysis was due to a bacteriocin or a phage, the VW28 supernatant was titred on G18. The results of the first experiment suggested that extremely low levels of phage were released since only 2×10^1 - 5×10^1 pfu/ml. could be detected. The plaques were very large (5mm. diameter), turbid, and without a halo. Attempts to form plaques on VW28 were unsuccessful. Therefore VW28 is lysogenic for a phage which plates on G18.

A single plaque was purified on G18 and a higher titre lysate of 10^8 pfu/ml. was produced. The turbidity of the plaques proved to be due to the growth of lysogens in the plaque itself. The phage was designated ϕ S28 and the lysogens G18(S28). The lysogens were isolated, from the growth from the centre of a turbid plaque, by streaking out onto a GSYC plate for isolated colonies. Several colonies were tested for lysogenic status by the standard spot test method, against ϕ S28 and culture supernatants from each test clone. The G18 (S28) lysogens showed the classical lysogenic response in releasing ϕ S28, which plated on G18, and being ϕ S28 resistant. Such G18 (S28) lysogens were stable as lysogens after six subcultures.

Table 4.3

Screen for phage/bacteriocin release

SUPERNATANT	TESTER RECIPIENT HOST				G18	CC276	NZP7
	W19	Su297	Su298	Nu18			
VW3	-	-	-	-	-	-	-
VW5	-	-	-	-	-	-	-
VW6	-	-	-	-	-	-	-
VW7	-	-	-	-	-	-	-
VW9	-	-	-	-	-	-	-
VW10	-	-	-	-	-	-	-
VW11	-	-	-	-	-	-	-
VW12	-	-	-	-	-	-	-
VW13	-	-	-	-	-	-	-
VW14	-	-	-	-	-	-	-
VW15	-	-	-	-	-	-	-
VW16	-	-	-	-	-	-	-
VW18	-	-	-	-	-	-	-
VW19	-	-	-	-	-	-	-
VW22	-	-	-	-	-	-	-
VW24	-	-	-	-	-	-	-
VW25	-	-	-	-	-	-	-
VW27	-	-	-	-	-	-	-
VW28	-	-	-	-	TL	-	-
VW29	-	-	-	-	-	-	-
VW31	-	-	-	-	-	-	-
VW32	-	-	-	-	-	-	-
VW33	-	-	-	-	-	-	-
VW36	-	-	-	-	-	-	-
VW38(1)	-	-	-	-	-	-	-
VW38(2)	-	-	-	-	-	-	-
VW51	-	-	-	-	-	-	-
VW53	-	-	-	-	-	-	-
VW54	-	-	-	-	-	-	-
VW56	-	-	-	-	-	-	-
VW57	-	-	-	-	-	-	-

Table 4.3 (contd)

	TESTER RECIPIENT HOST						
SUPERNATANT	W19	Su297	Su298	Nu18	G18	CC276	NZP7
VW58	-	-	-	-	-	-	-
VW59	-	-	-	-	-	-	-
VW60	-	-	-	-	-	-	-
Ø8.297	-	TL	TL	TL	-	-	-
Øi.NU18	-	-	TL	TL	-	-	-
Ø7.297	-	TL	TL	TL	-	-	-
ØI	CL	-	-	-	CL	CL	CL
ØC	CL	-	-	-	CL	CL	CL

TL = turbid lysis

CL = clear lysis

- = no lysis

Table 4.4Effect of various supernatants on W19

SUPERNATANT	RESULT	SUPERNATANT	RESULT
VW1	-	VW61	-
VW2	-	VW62	-
VW8	-	VW63	-
VW20	-	VW64	-
VW21	-	VW65	-
VW23	-	VW66	-
VW34	-	VW67	-
VW39	-	VW68	-
VW40	-	ØC.W19	C.L.
VW52	-	ØI.W19	C.L.
VW55	-	VW28	-

C.L. = clear lysis

- = negative

At this stage the release of ØS28 from VW28 was re-examined since, in a turbid spot test, it is not impossible that a bacteriocin is also released with the phage, but it is undetected in such an environment unless concentrated relative to the phage titre. Six different cultures of VW28 were grown to high cell density and their supernatants tested on a variety of strains by spot test. If a bacteriocin is released by VW28, and if G18 is sensitive to this bacteriocin, then it should be detected on the G18 (S28) lysogens which are ØS28 resistant. The results are presented in Table 4.6.

The data show that the detection of ØS28 on G18 is an irregular event because only 50% of the culture supernatants of VW28 release sufficient phage to be detected on G18. In contrast the G18 (S28) lysogen releases detectable levels of phage and such an effect is repeatable. It is interesting that W19 appears slightly sensitive to ØS28 when the ØS28 titre is boosted on G18 or released from a G18 (S28) lysogen. However the supernatant from VW28 has no effect on W19. Hence G18 is more sensitive than W19 to ØS28. This is exactly the opposite to the situation with ØC where W19 is the preferred host. VW28 supernatants do not give any lysis of G18 (S28) lysogens and so VW28 does not release a bacteriocin to which G18 is sensitive. Because ØS28.G18 gave a slightly turbid spot on W19 this interaction was investigated further.

Is the turbid spot reaction due to capsule depolymerase activity, bacteriocin or ØS28 ?

The results from the titration of ØS28.G18 on various hosts are presented in Table 4.7. The data show that plaques are indeed formed on W19 by ØS28 but they were very irregular. The phage also formed very small and irregular plaques on another strain of R.trifolii, namely NZP7. In an attempt to repeat the assays in the reverse direction

Table 4.5

Sensitivity of G18 to VW28 supernatant

TESTER STRAIN	VW28 ^S	G18 ^S	ØC
VW28	-	-	C.L.
G18	T.L.	-	C.L.

T.L. = turbid lysis

C.L. = clear lysis

- = no lysis

Table 4.6

Phage S28 release from lysogens

TESTER STRAIN	ØS28.G18	G18(S28) ^S	VW28S ¹	VW28S ²	VW28S ³	VW28S ⁴	VW28S ⁵	VW28S ⁶
G18	TL	TL	0	5TP	0	2TP	6TP	0
G18(S28)	0	0	0	0	0	0	0	0
W19	STL	STL	0	0	0	0	0	0
VW28	0	0	0	0	0	0	0	0

STL = slight turbid lysis

TL = turbid plaques

Table 4.7

Titration of ØS28.G18 on various hosts

HOST	TITRE (pfu ml ⁻¹)	e.o.p.
G18	1.2x10 ⁸	1
W19	3.4x10 ⁶ PP	2.8x10 ⁻²
NZP7	2.4x10 ⁵ PP	2.0x10 ⁻³
G18R ⁺ rif ^R	1.0x10 ⁸	0.83
G18SR	1.3x10 ⁸	1.08

PP = pin point plaques

G18SR = G18 str, rif

(i.e., ØS28.W19 titred on G18) the phage had to be passaged through W19 several times before a "workable" plaque morphology was obtained. This phenomenon also occurred with the passage of ØS28 through NZP7. The new plaque types were designated ØS28fp.W19 and ØS28fp.NZP7 respectively, because of the phages ability to produce "fuzzy" edges to the plaques. Instead of being pin-point these plaques were about 2mm in diameter. This form of selection of a plaque type which is easier to work with in a new host is analagous to the selection of Plkc, from phage Pl itself, in E.coli (Lennox, 1955).

It was hoped that ØS28 could be used as a vector for rhizobial DNA in future transduction experiments (Chapter 6), perhaps for the transduction of R-factor DNA. Therefore it was necessary at this stage to find out if the presence of the R-factor, R68.45, or indeed the genetic markers rif and str, had any effects on the replication of ØS28 in the R⁺ host. Such interference with phage replication has been shown to exist, in other genera, due to extra chromosomal elements (Van Emben et. al., 1976). From the data presented in Table 4.7 there is no evidence of any deleterious effects of this nature.

Titration of ØS28fp phages

The results, presented in Table 4.8, show that passage of ØS28 through W19 and NZP7 did not decrease the ability of the phages to form plaques on G18. These plaques, though, did tend to exhibit two phenotypes on G18. Of 104 plaques of ØS28fp.W19 on G18, 33 had a clearer edge and centre than the others which were more turbid and had a less well defined edge. This phenomenon was not investigated any further. There was no evidence of restriction of ØS28.

While propogating these phages it was obvious that ØS28 lysates tended to drop rapidly in titre e.g., over 5 weeks, at 4°C over CHCl₃, the titre of ØS28.G18 dropped by 5 logs !

Table 4.8

Efficiency of plating of ØS28 derivatives on W19 and G18

ØS28fp.W19			ØS28fp.NZP7		
HOST	pfu/ml	e.o.p.	HOST	pfu/ml	e.o.p.
W19	8.1×10^8	1	NZP7	1.2×10^6	1
G18	1.4×10^9	1.74	G18	2.0×10^6	1.6

Table 4.9

Inducibility of ØS28

HOST SUPERNATANT	UNTREATED (pfu/ml)	+ MITOMYCIN-C	
		PRE-DIALYSIS (pfu/ml)	POST-DIALYSIS (pfu/ml)
VW28	2×10^1	6×10^1	2×10^2
G18	N.D.	10^1	10^1
G18(S28)	2×10^3	3×10^5	5×10^5

N.D. = none detected

Inducibility of ϕ S28 from VW28 and G18(S28)

Cultures were induced as described in the general materials and methods section. Supernatants from the untreated culture and the induced culture were titred on G18. The supernatants from the induced cultures were also dialysed and then titred on G18. The results are presented in Table 4.9.

The data show that VW28 released low levels of ϕ S28 but mitomycin C treatment did not increase phage release to a significant extent. However, in G18(S28) lysogens, ϕ S28 was released at a detectable level and this release was stimulated by about 2 logs, after mitomycin treatment. Although ϕ S28 was induced in G18(S28) lysogens the titres of such lysates were not particularly high. Of course, it is possible that the mitomycin concentration of 0.5 μ g/ml. was not adequately high to give effective lysis and phage release.

Because it had proved to be easy to isolate G18(S28) lysogens by the standard techniques attempts were made to isolate ϕ S28fp lysogens of W19 and NZP7. All attempts were unsuccessful but the reasons for this are unknown.

It was hoped that ϕ S28 could prove to be effective as a transducing phage. Consequently a U.V. inactivation experiment was conducted to provide an estimate of the size of the ϕ S28 genome. Initial attempts at U.V. inactivation with the system used for the ϕ 7-related phages (Chapter 5) produced such rapid inactivation that between 3 and 4 logs of p.f.u. had been lost after 5 seconds exposure. Therefore lower U.V. doses were used in the inactivation of ϕ S28. Rhizobiophages C and I have a known genome size of 1.3×10^8 and 1.6×10^8 respectively (Atkins and Avery, 1974). Because ϕ S28 and ϕ C can plate on the same host, G18, then the sensitivity of both phages to U.V. can be compared under identical conditions. The results of U.V. inactivation of various phages are presented in Table 4.10 and Figure 4.1.

Table 4.10

U.V. Inactivation of various phages

PHAGE	EXPOSURE (SECS)	TITRE	SURVIVAL (tx"/to")
ØS28 (A)	0	1×10^6	1^{-1}
	5	3.3×10^5	3.3×10^{-1}
	10	1.5×10^5	1.5×10^{-1}
	20	2.6×10^4	2.6×10^{-2}
	40	6.0×10^2	6.0×10^{-4}
ØC.G18. W19 (B)	0	2.1×10^6	1^{-1}
	10	4.6×10^5	2.2×10^{-1}
	20	1.6×10^5	7.6×10^{-1}
	40	1.6×10^4	7.6×10^{-3}
	60	3.2×10^2	1.5×10^{-4}
ØC.G18. W19 (C)	0	3×10^7	1^{-1}
	10	7.2×10^6	2.4×10^{-1}
	20	2.0×10^6	6.7×10^{-2}
	40	2.5×10^5	8.3×10^{-3}
	60	6.0×10^3	2.0×10^{-4}
ØI.W19 (D)	0	4×10^6	1^{-1}
	10	1.1×10^6	2.8×10^{-1}
	20	4.8×10^5	1.2×10^{-1}
	40	4.8×10^4	1.2×10^{-2}
	60	4.4×10^3	1.1×10^{-3}

A = ØS28.G18 -----> G18
 B = ØC . W19 -----> G18
 C = ØC . W19 -----> W19
 D = ØI . W19 -----> W19

Conditions were standard (See general materials and methods) except irradiation was at 507 nm.

Fig.4.1 U.V. inactivation of various phages.

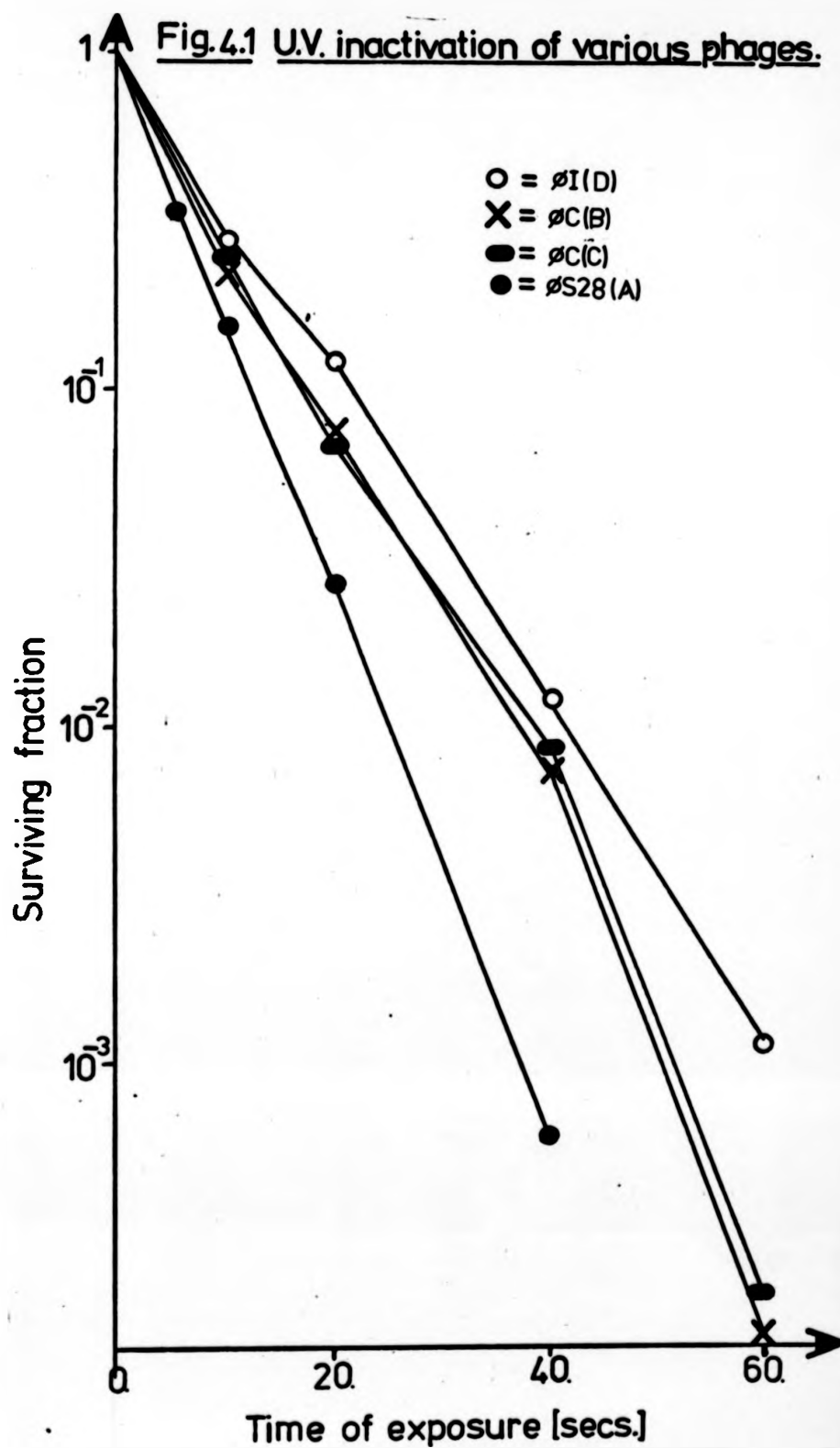


Figure 4.1 reveals that the host range mutant of ϕC , namely $\phi C.G18.W19$, is equally susceptible to U.V., as its wild type parent $\phi C.W19$. It is therefore a reasonable comparison to compare the U.V. inactivation rates of $\phi S28.G18$ and $\phi C.G18.W19$ as titrated on G18. It would appear that $\phi S28$ must be a large phage because it was inactivated more rapidly than ϕC . However, ϕI was inactivated less rapidly than ϕC under these conditions. It is known, from more accurate calculations, that ϕI is actually larger than ϕC (Atkins and Avery, 1974) and so ϕI should be more sensitive to U.V. inactivation, than ϕC , according to target theory (See Hayes, 1968). It is possible that ϕI has a genetic capability to partially reverse U.V. damage, but it appears more likely that this inconsistency between theory and practice is due to the variability of the experimental procedure used here. In summary, $\phi S28$ must have a large genome because it is U.V. inactivated as rapidly as ϕC .

CHAPTER V

VIRUS-HOST INTERACTIONS IN THE SU297/SU298 SYSTEM

"There's nocht that Science yet's begood to see
In hauf its deemless detail or its destiny.
Oor een gi'e answers based on pairt-seen facts
That beg a' questions, to ebb minds' content,
But hoo a'e feature or the neist attracts,
Wi' millions mair unseen, wha kens what's meant
By human brains and to what ends may tell
- For naething's seen or kent that's near a thing itsel'!"

Hugh MacDiarmid

"And what you do not know is the only thing you know"

TS Eliot

"But I can form nae notion o' the spirit
That gars it tak' the difficult shape it does"

Hugh MacDiarmid

Lysogeny in Rhizobium was originally discovered by Marshall in 1956. Marshall found that when two closely related strains of R. trifolii, Su297 and Su298, were mixed together in yeast-mannitol agar, plaques arose. The phage from these plaques could continue to form plaques on Su297 whereas the Su298 was resistant to the phage. Marshall suggested that Su298 was lysogenic for a phage for which Su297 was an indicator strain. However, subsequent research revealed that the relationship between Su297 and Su298 was more complex than Marshall had originally supposed.

Takahashi and Quadling (1961) discovered that Su297 released a phage-like particle after ultra-violet light treatment. This phage was designated ϕ_i and it could not form plaques on Su298. However, lysates of u.v.-induced Su297 were capable of inducing the development of two distinct phages in Su298. The two phages released were called ϕ_9 and ϕ_{10} (subsequently referred to as ϕ_7 and ϕ_8 by Barnet, 1968), and they could form plaques on Su297 and Su298 respectively. Both phages were considered to be serologically indistinguishable but distinct from ϕ_i . After u.v. treatment of Su298 lysis occurred but neither plaque forming phage nor phage-like particles could be detected by spot tests or electron microscopy. Consequently, Su298 was considered to be lysogenic for a defective prophage. Based on these discoveries, Takahashi and Quadling proposed that the two "new" phages could be generated in Su298 by two possible pathways. If ϕ_i DNA could recombine with the DNA from the defective prophage in Su298 then two novel phages could be generated. Alternatively, ϕ_i could act as an inducing agent in Su298 thereby releasing two phages, resident in Su298, by a mechanism not involving genetic exchange (Takahashi and Quadling, 1961).

Subsequent research showed that Su297 is, at least, bilysogenic and that the two phages released by Su297 can be distinguished by size, as

determined by electron microscopy (Barnet, 1968), Barnet showed that it is the smaller of the two phages, $\phi_i(s)$, which has the ability to induce ϕ_7 and ϕ_8 from Su298. Contrary to the results of Takahashi and Quadling, Barnet found that $\phi_i(s)$ and ϕ_7 are serologically indistinguishable. Barnet also proposed that ϕ_8 arises as a virulent mutant of $\phi_i(s)$. Evidence was also presented that suggested that restriction and modification of ϕ_8 operates between Su297 and Su298. Barnet also suggested that ϕ_7 is subject to restriction between Su297 and a strain of R. trifolii called Nul8. Although Barnet was unable to detect plaques on Su298 when Su298 was tested against ϕ_7 it was claimed that ϕ_7 vir could plaque on Su298. How ϕ_7 vir was isolated is a complete mystery since the conventional method of isolating a virulent mutant, by demonstrating its ability to plaque on a lysogen of the homologous, temperate phage, was not available. This is because Su297(7) lysogens are under the influence of phage conversion and are resistant to ϕ_7 , ϕ_8 and a clear plaque mutant of ϕ_7 , called ϕ_7^{cr} (Barnet, 1968; Barnet and Vincent, 1970).

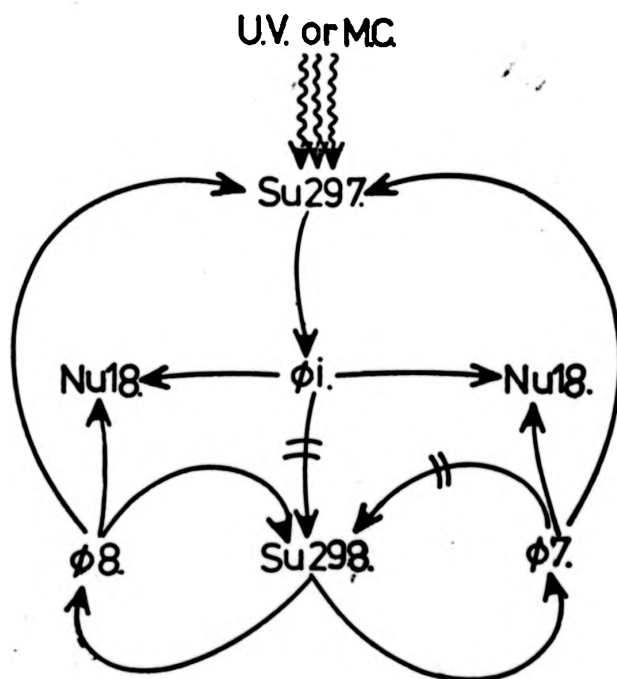
One of Barnet's most important findings was that $\phi_i(s)$ and ϕ_7 can form plaques on a third strain of R. trifolii called Nul8. Prior to this discovery it could not be proved that $\phi_i(s)$ was not defective because there was no host on which it could be plaque purified. Because $\phi_i(s)$ could not be purified away from Su297 it was also impossible to prove that $\phi_i(s)$ was responsible for "induction" of ϕ_7 and ϕ_8 . The discovery of Nul8 then, meant that the phages could be plaque purified away from their respective Su297 and Su298 hosts and the inductive power of $\phi_i(s)$ could be proved using purified phage. The general findings of Barnet (1968) and Takahashi and Quadling are summarised in Table 5.1, and the relationships between Su297, Su298 and Nul8 are depicted in Figure 5.1.

Because ϕ_8 is able to plaque on Su297 and Su298 it must be hetero-

Table 5.1 Summary of knowledge of the Su297/Su298 system

Takahashi and Quadling(1961)	Barnet(1968)
1. No free phage in Su298 lysates after u.v. irradiation.	No free phage in Su298 lysates after u.v. irradiation.
2. Su297 filtrates induce phage in Su298.	Su297 filtrates induce phage in Su298.
3. ϕ_i is the inductive agent.	Su297 is lysogenic for at least two phages. The smaller phage ($\phi_i(s)$) is the inductive agent.
4. ϕ_i doesn't plaque on Su298 and can't be purified because there is no alternative host.	ϕ_i doesn't plaque on Su298 but can be plaque purified on Nul8.
5. ϕ_i is u.v. and mitomycin inducible.	ϕ_i is u.v. inducible.
6. Thermal inactivation and u.v. inactivation curves of ϕ_i and ϕ_7 are similar.	The u.v. inactivation rates of ϕ_7 and ϕ_8 are slightly different.
7. ϕ_7 and ϕ_8 are serologically indistinguishable but these phages were grown on Su297 and would have been contaminated by ϕ_i .	ϕ_7 , ϕ_8 and ϕ_i are serologically indistinguishable.
8. ϕ_8 may be a virulent mutant of ϕ_7 .	ϕ_8 may be a virulent mutant of ϕ_i .
9.	Host controlled restriction and modification operate between Su297, Su298 and Nul8.
10.	ϕ_7 lysogenically converts Su297 to multiple phage resistance.
11.	ϕ_7 spontaneously mutates to ϕ_7^{cr} at a frequency of 10^{-3} .
12.	ϕ_7^{cr} forms clear plaques on Su297 but turbid plaques on Nul8.
13.	ϕ_7^{cr} is identical to ϕ_7 in every respect except the clear plaque phenotype.
14. ϕ_8 cannot plaque on Su297.	ϕ_8 can form plaques on Su297 but no lysogens can be isolated.
15.	No lysogens of Nul8 can be isolated for ϕ_i or ϕ_7 .
16.	Host range mutants of ϕ_7 are selected on Nul8 and such mutants cannot adsorb to Su297.

Fig.51 Summary of Su297/Su298 system.



————→ = forms plaques.

———//——→ = cannot form plaques.

U.V.=ultraviolet light.

M.C.=mitomycin-C.

immune with ϕ_i and any prophage(s) present in Su298. Correspondingly, because ϕ_7 is able to plaque on Su297 it has to be heteroimmune with ϕ_i . Because Su297(7) lysogens are subject to lysogenic conversion it was not possible to test if ϕ_8 and ϕ_7 were hetero or homoimmune. Hence any model to explain the formation of ϕ_8 and ϕ_7 from ϕ_i and Su298 has to account for the heteroimmunity of ϕ_i and ϕ_7 , and ϕ_i and ϕ_8 . Although Takahashi and Quadling only considered two rather vague models for ϕ_7 and ϕ_8 formation Barnet suggested a variety of possibilities. Barnet proposed that ϕ_i was incapable of generating two heteroimmune phages in Su298 unless there was a prophage already in Su298. Such a hypothetical prophage could be, as Barnet proposed, either defective, non-defective or very stable, and could interact with ϕ_i in the following ways:-

1. Su298 prophage defective

The defect in the Su298 prophage (ϕx^d) could be an absolute defect such as a deletion in essential genes. Alternatively, the defect could be due to an amber mutation(s). In the latter case the defect could be suppressible in a host carrying an amber suppressor. However, in either case, infective phage could be expected to be formed by recombination between ϕ_i and ϕx^d . It would also be possible to generate infective phage by a process of phenotypic mixing, e.g. the packaging of ϕx^d DNA in ϕ_i coat proteins. In the latter event it would also be possible to envisage ϕ_7 , formed by such phenotypic mixing, multiplying in Su297 due to induction and expression of ϕ_i genes. Another possibility could be that ϕ_i could act as a superinfecting helper phage for ϕx^d by providing specific functions such as enzymes of DNA replication necessary for the production of mature phage. Finally, although the ϕ_7 generated from ϕ_i and the defective ϕx^d could be carrying an amber mutation(s), it may be able to replicate only on Su297 because Su297 could carry an amber suppressor which is absent from Su298.

2. Su298 prophage not defective

If the prophage in Su298 is not defective then there would have to be some alternative reason for its inability to plaque on Su297, or Nul8. Perhaps host range mutants could be formed by recombination, between ϕ_i and a vegetative ϕ_x , or by phenotypic mixing. However, in the latter case phage could multiply in Su297 only if the loss of infectivity of ϕ_x in Su297 was restricted to a process determined by the coat proteins e.g. adsorption or penetration.

3. Su298 prophage very stable

Perhaps u.v. and mitomycin-C cannot induce the ϕ_x prophage because it is "ultrastable". Hence ϕ_i could induce ϕ_x only if it could provide some extra "induction shock" or "superinduction".

Alternatively the prophage may lack the genes needed for prophage excision and ϕ_i could supply such function(s).

All of these possibilities could be grouped under one of two models. It is possible to view the biogenesis of ϕ_7 and ϕ_8 as determined principally by a recombinational or non-recombinational event. Hence, if an exchange of genetic elements is required for ϕ_7 and ϕ_8 formation, a recombinational model would be adequate to explain the synthesis of these phages. However, it is possible that no recombination occurs in the synthesis of ϕ_7 and ϕ_8 and in this instance a physiological or epigenetic model could be used to explain the formation of the phages. Finally, it is also possible that the formation of ϕ_7 and ϕ_8 cannot be adequately explained by either model in isolation but only by a combination of both.

Why study the Su297/Su298 system?

It is surprising that since 1956 this system has only been investigated by two groups of workers and therefore has remained largely unexploited in viral, genetic and molecular biological terms. From the information in Table 5.1 it can be seen that several well known phenomena of molecular biology e.g. phage conversion; restriction and modification; host range mutant selection and phage evolution, are present in this system. When one considers that this is in a genus for which there is very little detailed molecular knowledge then it becomes even more surprising that this system has remained under-exploited!

As mentioned in the general introduction, the fields of rhizobial genetics and molecular biology have been characterised by inadequately controlled experiments. Hence, in such a complex system as the Su297/Su298 system, it is of crucial importance to demonstrate that the results of earlier preliminary experiments by Barnet, and Takahashi and Quadling are repeatable.

Consequently in this short study the first experiments conducted were an attempt to define the bacterial strains; the phages they release and the relationships between phage and host. Based on the results from such experiments further research was undertaken to try to discover more about the phage-host interactions in the system and to test the predictions which arise naturally as a consequence of invoking the recombinational model for $\phi 7$ and $\phi 8$ formation.

Material and Methods

Most materials and methods relevant to this section are in the general materials and methods section. Additional pertinent methods are listed below.

Ultraviolet Inactivation of Rhizobiophage

Phage lysates were diluted 1:100 into phage buffer. About 5ml. of this diluted lysate was poured into a sterile glass petri-dish such that the bottom surface of the dish was just covered by the liquid. The surface of the liquid was irradiated with u.v. light at a distance of 220mm from the source of a short wavelength emission tube (Anderman and Co., London). The lamp was always switched on thirty minutes before use, to allow equilibration, and the glass dish was agitated throughout the irradiation. Lysate samples of 0.1ml were removed at intervals and serially diluted into phage buffer before titration on the appropriate host. Plaques were counted after 2 days at 30°C.

Thermal Inactivation of Rhizobiophage

Phage lysates were diluted 1:10 into phage buffer in preheated, thin-walled, sterile, glass test tubes which were maintained at 60°C \pm 0.5°C. in a water bath. Where several lysates were being subjected to heat treatment glass test-tubes of the same thickness were used. Samples from the lysates were removed at various time intervals and serially diluted into ice-cooled tubes containing phage buffer. From these ice-cooled tubes samples were titred on the appropriate hosts.

Rhizobiophage Concentrations

Phage lysates were concentrated by the polyethylene glycol 6000 method of Yamamoto *et al.* (1970) using polyethylene glycol 6000 at 10% (v/v) and NaCl at 0.5M.

Caesium Chloride Density Gradient Centrifugation

CsCl was dissolved in distilled water or phage buffer and step gradients were prepared of 1.3, 1.4, 1.5 and 1.6 g/c.c. using 0.5ml or

1.0ml of each density, depending on the tube size used. Phage lysates, concentrated by the Yamamoto method, were carefully layered onto the gradient and centrifuged in a 3 x 6.5ml titanium rotor at 35,000 r.p.m. (MSE 65) at 4°C for 2-3 hrs. After each run the tubes were carefully removed and examined for the characteristic opalescence of a phage band, using top illumination against a black background.

Metrizamide Density Gradient Centrifugation

Metrizamide was prepared, in distilled water at pH7, as 20,40,60 and 80% w/v solutions. Such solutions yielded densities of 1.11,1.22, 1.38 and 1.46 g/cc respectively, at 5°C. The centrifugation conditions were identical to those used for CsCl.

Ultrafiltration

Phage lysates of various volumes were filtered through XM300 membrane filters with a molecular weight exclusion limit of 300,000 daltons. All filtrations used the diaflo ultrafiltration cell with nitrogen gas to produce a top pressure of 8 p.s.i. at 4°C. Ultrafiltration was stopped when between 2 and 10ml of lysate were left above the membrane, when the filtrate was removed for titration. The flow rate of the lysate through the membrane was dependent on the initial viscosity of the lysate.

Lysogen Construction

Cultures were grown in GSYC broth or LSB to an O.D.₅₄₀ of 0.2 to 0.3, and top agar layers of GSYC were seeded with 0.2ml of the culture. When the top agar was set one drop of phage lysate at a titre of 10^8 pfu/ml was spotted onto the agar and allowed to dry. After incubation at 30°C for 2-3 days turbid lysis had occurred in the lysate spot, although the amount of bacterial growth in the spot test was phage dependent. A loopful of the turbid growth was streaked out, on GSYC agar, to well isolated colonies. Individual colonies were purified and used to inoculate 5ml of GSYC broth. These cultures

were then tested for the characteristics of a lysogen, namely, phage release; resistance to phage and stable inheritance of both phenomena. All such tests involved the standard spot test assay. Only where a culture released a phage to which it was itself immune was it considered lysogenic for that phage.

Production of Rhizobiophages

Øi

Phi i was produced initially by mitomycin-C (MC) induction of Su297 which is naturally lysogenic for Øi. Although it was not a pure lysate it was labelled Øi.Su297. The phage was plaque purified on Nul8. Purified Øi was labelled Øi.Nul8.

Ø7

Phi 7 lysates were produced in two ways.

1) Broth induction method

MC-induced Su297 lysates were added to early log phase cultures of Su298 at an m.o.i. of about 1, as titred on Nul8. The Su298/Øi mixture was incubated overnight at 30° before centrifugation and collection of the supernatant. This supernatant was chloroform sterilised and titred to isolated plaques on Su297. A large turbid plaque was purified on Su297 to make the lysate Ø7.Su297. This lysate was used to make a plaque purified Ø7 lysate, on Nul8.

2) Plate method

Plaque purified Øi.Nul8 was spot tested on Su298 to give turbid lysis. A loopful from the turbid spot was suspended in 1ml of phage buffer over a few drops of chloroform. This lysate was titred on Su297 to isolated turbid plaques. A representative plaque was chosen, and a plaque purified Ø7.Su297 was produced from this plaque.

Ø8

Phi 8 lysates were produced in two ways.

1) Broth induction method

This method was exactly the same as for the broth induction method of $\phi 7$ production. However, after overnight incubation of the Su298/ ϕi mixture, the chloroform sterilised lysate was titred on Su298 rather than on Su297. A representative plaque was chosen and used to produce a pure $\phi 8$.Su298 lysate.

2) Plate method

An MC-induced Su297 lysate or ϕi .Nu18 was titred on Su298. Plaques arose at low frequency. Representative plaques were purified on Su298. As shown in this study this phage, ϕi .Su298, has the same host range as $\phi 8$.

The phenotypic appearance of $\phi 7$ on Su297 and $\phi 8$ on Su298 conformed to the descriptions of these plaques as described by Takahashi and Quadling (1961), and Barnet (1968). Also, when $\phi 7$.Su297 was titred on Su297, clear plaques arose at a frequency of about 10^{-3} . These plaques were purified on Su297 and designated $\phi 7^C$.Su297. Barnet (1968) called such phage $\phi 7^{CR}$.

Results I.

1. Absolute requirement of ϕ i for the biogenesis of ϕ 7 and ϕ 8.

a) Which combinations of Su297 supernatant and Su298 lead to phage release?

Cultures and filter sterilised supernatants of Su297 and Su298 were combined in various ways and the resultant lysates spot tested on both hosts to assay for lysis. The results are presented in Table 5.2.

b) Effect of purified ϕ i on ϕ 7 and ϕ 8 release

Plaque purified ϕ i.Nul8 was spot tested on various hosts. Also, ϕ i.Nul8 was added to log phase cultures of various hosts and incubated for four hours. The resultant lysates were used in the spot test assays. The results are presented in Table 5.3.

2. Broth and plate method of ϕ 7 and ϕ 8 production

a) Broth method

Prewarmed Su297 supernatant, or ϕ i.Nul8 lysate, was added to an equal volume of an early log phase culture of Su298 and incubated for several hours. The increase in O.D. was followed at various time intervals and the results obtained using the Su297 supernatant are presented in Table 5.4 and illustrated in Figure 5.2. Using ϕ i.Nul8 instead of Su297 supernatant similar results were obtained (data not recorded).

The lysates were incubated at 30°C overnight then the cells were removed by low speed centrifugation. The supernatants were collected and stored over chloroform before titration on the various hosts. The results are presented in Table 5.5.

b) Plate method

Drops of ϕ i.Nul8 and Su297 supernatant were spotted onto top agar lawns of Su298 and incubated at 30°C to give turbid lysis. A small sample was punched out of the turbid lysis zone and suspended in 1ml of phage buffer, chloroform sterilised and titred on Su297

and Su298. The results are presented in Table 5.6.

3. Direct titration of Øi.Nul8 and Su297 supernatant on Su298 and other hosts

A lysate of Øi.Nul8 and the supernatant of Su297 was titred on Su297, Su298 and Nul8. The results are presented in Table 5.7.

Table 5.2

Lysis of Su297 and Su298 by various supernatants

INDICATOR	TESTER SUPERNATANT	LYSIS
Su297	Su297 ^s	-
Su297	Su298 ^s	-
Su297	(Su297 ^s + Su298 ^s)	-
Su298	(Su297 ^s + Su298 ^s)	T.L.
Su298	Su298 ^s	-
Su298	Su297 ^s	T.L.
Su297	(Su297 + Su298) ^s	T.L.
Su298	(Su297 + Su298) ^s	T.L.
Su297	(Su297 + Su298) ^{s*}	-
Su298	(Su297 + Su298) ^{s*}	T.L.
Su297	(Su298 + Su297 ^s) ^{s*}	T.L.
Su297	(Su298 + Su297 ^s) ^{s*}	T.L.

T.L. = turbid lysis

- = no lysis

s = supernatant

* = sterile supernatant from one culture was mixed in a 1:1 ratio to an early log phase culture of heterologous/homologous cells and incubated for at least four hours. Cells were then removed by centrifugation and the sterilised lysate used for the spot test assay.

Table 5.3

Effect of purified ϕ i on ϕ 7 and ϕ 8 release

INDICATOR	TESTER SUPERNATANT	LYSIS
Nul8	ϕ i.Nul8	T.L.
Su297	"	-
Su298	"	T.L.
Su297	(ϕ i.Nul8 + Su297) ^s	-
Su297	(ϕ i.Nul8 + Su298) ^s	T.L.
Su297	(ϕ i.Nul8 + Nul8) ^s	-
Su298	(ϕ i.Nul8 + Su297) ^s	T.L.
Su298	(ϕ i.Nul8 + Su298) ^s	T.L.
Su298	(ϕ i.Nul8 + Nul8) ^s	T.L.
Su297	Nul8 ^s	-
Su297	(Nul8 ^s + Su298) ^s	-
Su298	(Nul8 ^s + Su298) ^s	-

T.L. = turbid lysis

- = no lysis

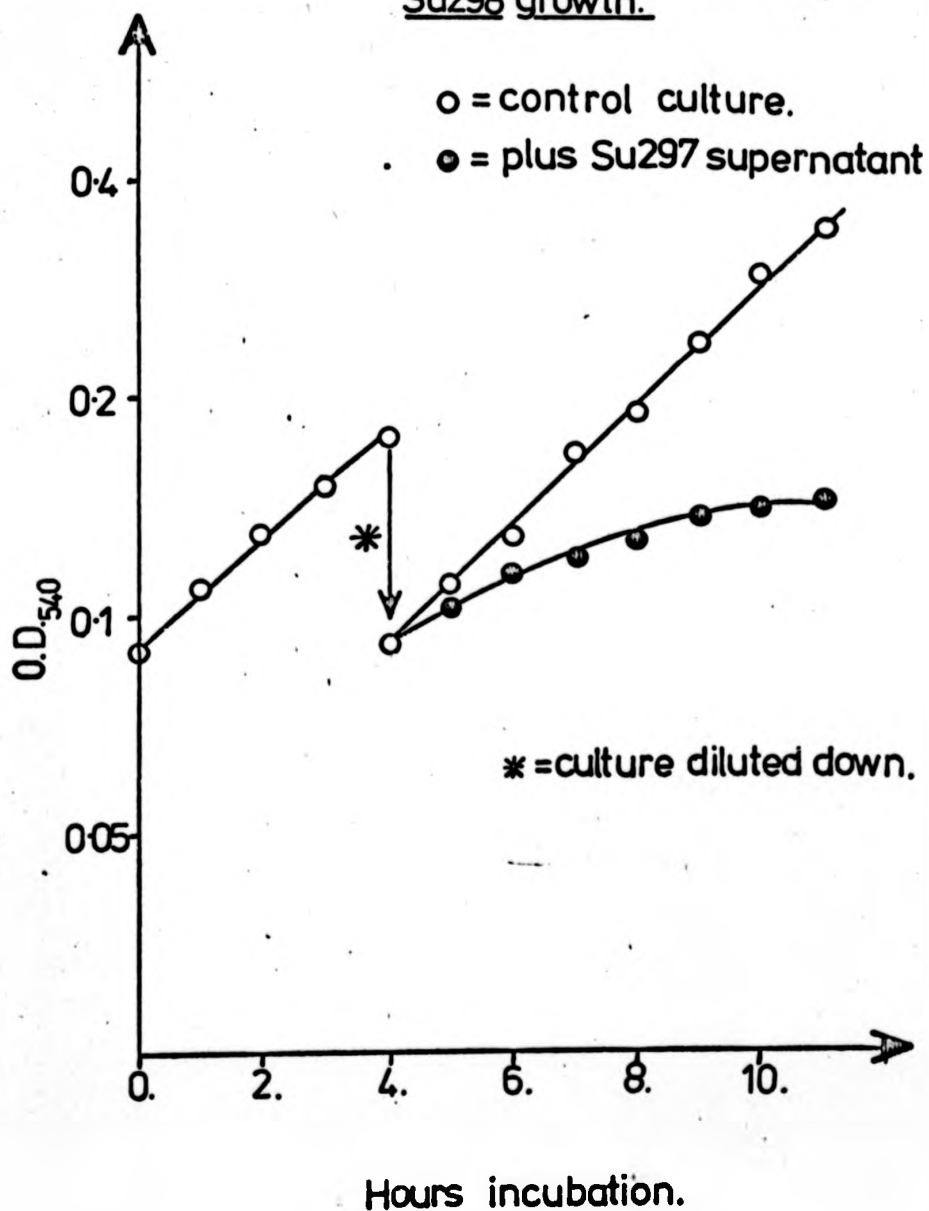
s = supernatant

Table 5.4Effect of Su297 supernatant on Su298 growth

Time post inoculation (hours)	O.D. 540	
	Control	plus Su297 supernatant
0	0.090	-
1	0.110	-
2	0.130	-
3	0.150	-
4	0.180	-
* 4	0.090	0.090
5	0.110	0.105
6	0.130	0.115
7	0.175	0.120
8	0.195	0.130
9	0.240	0.140
10	0.300	0.145
11	0.350	0.145

* = the culture was split into two equal portions at this point and an equal volume of prewarmed Su297 supernatant was added to one portion while the same volume of prewarmed G.S.Y.C. broth was added to the control culture.

Fig. 5.2 Effect of Su297 supernatant on Su298 growth.



Discussion

I. Absolute requirement of ϕ i for the biogenesis of ϕ 7 and ϕ 8

The results presented in Table 5.2 are in agreement with those found by Takahashi and Quadling (1961) and Barnet(1968). Su297 releases some agent which causes turbid lysis on Su298 lawns. Su297 is immune to this factor. However, Su298 supernatant has no noticeable effect on either Su298 cells or on Su297. Mixing of Su297 and Su298 supernatants did not give rise to any factor which caused lysis of Su297, yet, because of the presence of Su297 supernatant an effect was noticed on Su298. When viable cell cultures of Su297 and Su298 were mixed for several hours the resultant supernatant was capable of lysing both Su297 and Su298. However, although this effect can be repeated using Su297 supernatant and viable Su297 cells it cannot be repeated without viable Su298 cells. Therefore these data can be summarised by saying that for the production of turbid lysis on both Su297 and Su298 the test lysate must be made from viable Su298 cells and either Su297 supernatant or cells.

Although viable Su298 cells are an obligate requirement for turbid lysis on Su297 and Su298 it is possible that there is some chemical agent rather than biological agent released from Su297. Strain Nul8 is sensitive to Su297 supernatant but not Su298 supernatant. When Su297 supernatant is titred on Nul8 large (2-3mm diameter) turbid plaques arise (Barnet, 1968). This phage was called ϕ i(s) by Barnet. When this phage was purified on Nul8 then tested on the three hosts some interesting results were obtained (Table 5.3). In agreement with Barnet's results ϕ i gave turbid lysis of Su298 and Nul8 but not Su297. However when ϕ i.Nul8 was added to viable Su298 cells and incubated for four hours the resultant lysate was capable of lysing Su297. Nul8 supernatant was incapable of lysing Su297 as was a mixture of Nul8 and Su298 supernatants.

These experiments show that the key factors required for the

production of a lysate capable of lysing both Su297 and Su298 are ϕ_i and viable Su298 cells. Hence ϕ_i induces the formation of something in Su298 which can lyse Su297.

2. Broth and Plate method of ϕ_7 and ϕ_8 production

a) Broth method

A few hours after addition of Su297 supernatant to Su298 the OD₅₄₀ declined slightly as compared with the control culture. However no obvious lysis had occurred and although light microscopy showed very pronounced pleomorphism and slight debris there was no sign of "dramatic" lysis. In fact it appeared that all growth was inhibited rather than that the culture was lysing. Such observations are in agreement with the results of Takahashi and Quadling (1961). Four hours after addition of phage the cells and debris were removed by centrifugation and the sterilised supernatant was titred on Su297, Su298 and Nul8. The results are presented in Table 5.5.

Both Su297 supernatant and ϕ_i .Nul8 have an inductive capability. The phage(s) responsible for the plaques on Su297 is the more frequent of the two (ϕ_7 and ϕ_8). This phage has been called ϕ_7 by Barnet and the phage which plaques on Su298 has been called ϕ_8 . The data show that ϕ_7 is about 5×10^4 times more abundant than ϕ_8 after induction. An interesting point is that ϕ_7 plates on Su297 more efficiently than it does on Nul8. The plaques on Nul8 could be due to ϕ_7 , ϕ_8 and residual ϕ_i which had not been adsorbed to Su298 cells. Whatever is occurring here it does mean that, irrespective of the character of the phage plaquing on Su297, if it does plaque on Nul8 it does so with a lower e.o.p. than on Su297.

The plaques formed on Su298 could have come from three sources.

Table 5.5Broth method induction of Ø7 and Ø8

INDUCING AGENT ON Su298	TITRE OF FINAL LYSATE ON		
	Su297	Su298	Nu18
Su297 SUPERNATANT	5.2×10^6	9.0×10^1	N.T.
Øi.Nu18	5.0×10^5	1.0×10^1	1.0×10^3

N.T. = not tested

Either they are mutants of ϕ i or they are "new" phages formed by the passage of ϕ i through Su298. According to Barnet (1970) ϕ i can adsorb to Su298 but it has never been proved that ϕ i injects its nucleic acid into Su298. Consequently, although highly unlikely, it is not impossible that the plaques on Su298 are due to some effect of ϕ i whereby it can "induce" some resident pseudolysogenic prophage in Su298 which can then plaque on the same host.

b) Plate method

Although turbid lysis occurred with ϕ i.Nul8 or Su297 supernatants on Su298 lawns the phage present only plaqued on Su297. This seems remarkable since one would expect re-lysis of Su298 by free phage. This implies that the phage causing the lysis of Su298 cannot complete the full replicative cycle on Su298. As with the data from the broth induction experiments, ϕ 7 was the dominant phage after lysis of Su298. It is possible that ϕ 8 was present in the spot test but that the probability of detecting it is of the order of 10^{-4} of the frequency of ϕ 7 detection. Therefore, because low numbers of plaques were detected on Su297 in these plate method experiments, it is not surprising that plaques were not detected on Su298.

3. Direct titration of ϕ i and Su297 supernatant on Su298 and other hosts

The results show that ϕ i lysates can indeed give rise to plaques on Su298. However these plaques arise at low frequency i.e. about 10^{-4} for Su297 supernatant, relative to the Nul8 titre, and about 10^{-6} for ϕ i.Nul8. Interestingly, there was no correlation between the dilution factor and the plaque number when ϕ i.Nul8 was titred on Su298. This effect was always repeatable. Why there should be no proportionality here is unknown, but it suggests that the formation of plaques on Su298 is not a direct reflection of the ϕ i titre alone. It seems reasonable to ask then, to what are these plaques due? If

Table 5.6Plate method induction of Ø7 and Ø8

INDUCING AGENT ON Su298	TITRE ON	
	Su297	Su298
Su297 SUPERNATANT	6.0×10^2	N.D.
Øi.Nu18	3.0×10^3	N.D.

N.D. = no plaques detected

Table 5.7Titration of Øi.Nu18 and Su297 supernatant

RECIPIENT	"LYSATE" USED	
	Øi.Nu18	Su297 SUPERNATANT
Su297	N.D.	N.D.
Su298	2.9×10^3 *	2.5×10^3
Nu18	3.0×10^9	1.7×10^7

N.D. = no plaques detected

* = this titre is the average of plaque numbers from four plates i.e. Neat 14 & 16; 10^{-2} 2 & 8. (See text for discussion).

they were just ϕ_i plaques then they should not be able to plaque on Su297 due to immunity conferred by the ϕ_i resident prophage. Correspondingly, unless Su298 is a pseudolysogen for this phage, the plaques must have been formed by a phage which is heteroimmune with any phages which may be resident in Su298. Although Barnet noted that high titre ϕ_i lysates could form plaques on Su298 (Barnet, 1968) she failed to analyse the characteristics of such phage. Hence it was considered important to investigate the host range of these phages in the hope that such a study would cast some light on the biogenesis of ϕ_7 and ϕ_8 .

Conclusions

These experiments have proved that the basic data of previous workers is correct and repeatable. ϕ_i is an obligate requirement for the biogenesis of ϕ_7 and ϕ_8 . Also, ϕ_i can form plaques on Su298 but with a low e.o.p. which is not proportional to the titre of the lysate. The phage in such plaques are examined in later sections.

RESULTS II.

Construction and phage sensitivity of lysogens

1. Lysogens of Su297

Su 297 is naturally lysogenic for ϕ i (Barnet, 1968; this study) and consequently is immune to superinfection by this phage. Attempts were made at isolating ϕ 7, ϕ 7^c and ϕ 8 lysogens of Su297 by the standard methods. Although it was not quantified lysogens were isolated with high frequency and the results of typical responses to the phage release and sensitivity tests are presented in Tables 5.8 and 5.9.

2. Lysogens of Su298

By the standard methods attempts were made at isolating Su298 lysogenic for ϕ i, ϕ 7, ϕ 7^c and ϕ 8. For each phage tested, twenty colonies of putative lysogens were assayed for phage release and phage sensitivity. On no occasion could lysogeny be demonstrated.

3. Lysogens of Nul8

Attempts were made at isolating ϕ i, ϕ 7 and ϕ 8 lysogens of Nul8. On several occasions some colonies yielded the phenotype expected of a true lysogen but when re-examined after several subcultures such clones always appeared to revert to the non-lysogenic state. On two occasions it proved possible to isolate ϕ i and ϕ 7 resistant colonies. The results of phage sensitivity and phage release tests on representative colonies from these assays are presented in Tables 5.10, 5.11 and 5.12.

The data in Tables 5.8 and 5.9 are representative of the response of many isolates of ϕ 7, ϕ 7^c and ϕ 8 lysogens of Su297. All behaved in the classical fashion i.e. they all released the phage to which they were lysogenic and they were resistant to that phage. They were also stably lysogenic after five subcultures. There are several other features, however, which are of interest.

As shown by Barnet (1968), when ϕ 7 and ϕ 7^c lysogens of Su297 are

Table 5.8 Typical response of lysogens; phage release

Strains	SUPERNATANT			
	Su297	Su297(7)	Su297(7 ^c)	Su297(8)
Su297	-	T.L.	C.L.	T.L.
Su297(7)	-	-	-	-
Su297(7 ^c)	-	-	-	-
Su297(8)	-	-	-	-
Su298	S.T.L.	S.T.L.	S.T.L.	S.T.L.
Nu18	T.L.	T.L.*	T.L.	T.L.

S.T.L. = slight turbid lysis

* = low numbers of turbid plaques

C.L. = clear lysis.

T.L. = turbid lysis.

Table 5.9 Typical response of lysogens: phage sensitivity

STRAIN	LYSATE				
	Su297 Super- natant	Ø7.Su297	Ø7 ^c .Su297	Ø8.Su297	Øi.Nu18
Su297	-	T.L.	C.L.	T.L.	-
Su297(7)	-	-	-	-	-
Su297(7 ^c)	-	-	-	-	-
Su297(8)	-	-	-	-	-
Su298	S.T.L.	S.T.L.	S.T.L.	S.T.L.	T.L.
Nu18	T.L.	T.L.	T.L.*	T.L.	T.L.

* = low numbers of turbid plaques

S.T.L. = slight turbid lysis

tested for phage sensitivity, they exhibit cross resistance to $\phi 7$, $\phi 7^c$ and $\phi 8$. It has been shown that this is due to phage conversion (Barnet and Vincent, 1970). Contrary to the claims of Barnet however, it proved very easy to isolate $\phi 8$ lysogens of Su297. Moreover, the Su297($\phi 8$) lysogens reacted in exactly the same way as the Su297($\phi 7$) lysogens in their phage sensitivity patterns. This may mean that when $\phi 8$ lysogenises Su297 phage conversion occurs as with $\phi 7$. According to Barnet's model $\phi 8$ was a virulent mutant of ϕi and hence no Su297($\phi 8$) lysogens could be isolated. Either Barnet's $\phi 8$ is genetically distinct from the $\phi 8$ used here or her model for the formation of $\phi 8$ is incorrect. The fact that stable lysogens of $\phi 8$ can be isolated show that $\phi 8$ is heteroimmune with ϕi and therefore must be a genetically distinct phage from ϕi . It is interesting in this context that, even when high titre lysates of ϕi were tested against Su297, no plaques ever appeared. This means that ϕi^{vir} does not appear spontaneously in a ϕi lysate even with as low a frequency as 10^{-8} . Neither does it do so after extensive u.v. irradiation of ϕi lysates. Consequently, Barnet's model for $\phi 8$ formation cannot be correct.

All of the lysogens released phage which gave a slightly turbid lysis of Su298. Because they must all be naturally lysogenic for ϕi then it is impossible at this stage to say if the turbid lysis is due to ϕi or the other phages as well. An interesting point is that although Su297($\phi 7^c$) lysogens released phage which gave clear lysis of Su297 they only gave isolated turbid plaques when their supernatants were tested on Nu18. The reason for this is unknown at this stage.

No lysogens of Su298 were isolated. The reason for this is unknown but the results are in agreement with those of Barnet (1968). This means that, if Su298 can be lysogenised by any of these phages,

the event occurs at relatively low frequency. Alternatively, the prophages may not be stably maintained in an Su298 background.

The data in Tables 5.10 and 5.11 suggest that Nul8 can become "transiently" lysogenic for ϕ i, ϕ 7 or ϕ 8 during which time multiple phage resistance is expressed. However, two subcultures later, isolated colonies lost this phenotype and "reverted" to the phenotype of the parental Nul8. It may be that stable lysogens of ϕ 7 and ϕ 8 can only be formed in the presence of ϕ i or that neither Su298 nor Nul8 has the necessary prophage attachment sites for these phages so that the prophage is segregated out as the cells divide. Alternatively these clones could be phage resistant mutants which hold the phages in the "carrier" state of pseudolysogeny (Barksdale and Arden, 1974; Baess, 1971). However, this is very unlikely since one would not expect such derivatives to revert to phage sensitivity, as pseudolysogens or otherwise, with such a high frequency. If they are transiently lysogenic it is very interesting that, in Nul8, transient lysogeny with ϕ i confers multiple phage resistance whereas Su297(i) lysogens are ϕ 7, ϕ 7^c and ϕ 8 sensitive. Whatever the explanation for these results it does seem that the relationships between phage and host are very variable between Su297, Su298 and Nul8.

An interesting feature of these studies is that ϕ i and ϕ 7 resistant colonies of Nul8 were isolated. Such derivatives exhibited multiple phage resistance but, of course, they did not release any phage (Table 5.12). This suggests that ϕ i, ϕ 7 and ϕ 8 all have common receptor sites on the surface of Nul8.

Table 5.10 Examples of Nul8 clones recovered after attempts at isolating lysogens: Tested after 1 subculture

a) Phage release

TESTER	SUPERNATANTS			
	Nul8(i)	Nul8(7)	Nul8(8)	H ₂ O control
Nul8	T.L.	T.L.	T.L.	-
Nul8(i)	-	-	-	-
Nul8(7)	-	-	-	-
Nul8(8)	-	-	-	-

b) Phage sensitivity

TESTER	Øi.Nul8	Ø7.Nul8	Ø8.Nul8	H.S.	H ₂ O control
Nul8	T.L.	T.L.	T.L.	-	-
Nul8(7)	-	-	-	-	-
Nul8(8)	-	-	-	-	-
Nul8(i)	-	-	-	-	-

H.S. = homologous supernatant

- = negative

Table 5.11 Examples of Nul8 clones recovered after attempts at isolating lysogens: Tested after 3 subcultures

a) Phage release

TESTER	SUPERNATANT			
	Nul8(i)	Nul8(7)	Nul8(8)	H ₂ O control
Nul8	-	-	-	-
Nul8(i)	-	-	-	-
Nul8(7)	-	-	-	-
Nul8(8)	-	-	-	-

b) Phage sensitivity

TESTER	Øi.Nul8	Ø7.Nul8	Ø8.Nul8	H.S.	H ₂ O control
Nul8	T.L.	T.L.	T.L.	-	-
Nul8(7)	T.L.	T.L.	T.L.	-	-
Nul8(8)	T.L.	T.L.	T.L.	-	-
Nul8(i)	T.L.	T.L.	T.L.	-	-

T.L. = turbid lysis

- = negative

Table 5.12 Putative multiply resistant clones of Nul8

a) Phage release

TESTER	SUPERNATANT		
	Nul8(i)cl.15	Nul8(7)cl.21	H ₂ O control
Nul8	-	-	-
Nul8(i)cl.15	-	-	-
Nul8(7)cl.21	-	-	-

b) Phage sensitivity

TESTER	Øi.Nul8	Ø7.Nul8	Ø8.Nul8	C.
Nul8	T.L.	T.L.	T.L.	-
Nul8(i)cl.15	-	-	-	-
Nul8(7)cl.18	-	-	-	-

Inducibility of Su297 lysogens

The lysogens, isolated in the previous section, were tested for mitomycin-C inducibility because previous data showed that ϕ_1 , ϕ_7 and ϕ_7^C were inducible in Su297 (Takahashi and Quadling, 1961; Barnet, 1968). The results of the growth experiments in the presence and in the absence of mitomycin C, are recorded in Table 5.13 and illustrated in Figure 5.3.

Because the derivative of Su297 used throughout was the rough derivative of the smooth Su297 parent, and because previous plate assays had shown that the rough derivative was more sensitive to mitomycin (see Chapter Four) smooth and rough derivatives of non-lysogenic G18 and G15 were included to determine the effect of MC on growth, as opposed to MC stimulated phage induction.

After lysis was complete, the cells and debris were removed by centrifugation and the supernatants were dialysed against phage buffer to remove the MC. These lysates were then titrated on their respective hosts. The results are presented in Table 5.14.

Table 5.13 Growth of various strains in the presence of mitomycin C

STRAIN	HOURS POST SUBCULTURE	OPTICAL DENSITY AT 540nm		
		-M.C.	+ M.C. (0.5 μ g/ml)	+ M.C. (1.0 μ g/ml)
Su 297	0	0.074	-	-
	1	0.090	-	-
	2*	0.120	-	-
	3	0.150	0.150	-
	4	0.200	0.170	-
	5	0.270	0.150	-
	6	0.340	0.084	-
	7	0.440	0.055	-
Su297(7)	0	0.098	-	-
	1*	0.120	-	-
	2	0.170	0.170	-
	3	0.230	0.190	-
	4	0.270	0.210	-
	5	0.340	0.090	-
	6	0.440	0.060	-
	7	0.480	0.048	-
Su297(7 ^c)	0	0.080	-	-
	1	0.100	-	-
	2	0.130	-	-
	3*	0.170	0.170	-
	4	0.210	0.190	-
	5	0.270	0.210	-
	6	0.350	0.080	-
	7	0.420	0.050	-
Su297(8)	0	0.090	-	-
	1	0.110	-	-
	2	0.140	-	-
	3*	0.190	0.190	-
	4	0.250	0.230	-
	5	0.310	0.260	-
	6	0.390	0.250	-
	7	0.480	0.070	-
8		0.490	0.045	-

STRAIN	HOURS POST SUBCULTURE	OD540		
		-M.C.	+M.C. (0.5 μ g/ml)	+M.C. (1.0 μ g/ml)
G15	0	0.080	-	-
	1	0.098	-	-
	2*	0.120	0.120	0.120
	3	0.150	0.144	0.140
	4	0.190	0.180	0.160
	5	0.240	0.210	0.180
	6	0.300	0.240	0.190
	7	0.370	0.290	0.210
	8	0.440	0.330	0.230
G18	0	0.070	-	-
	1	0.082	-	-
	2	0.100	-	-
	3	0.120	-	-
	4*	0.150	0.150	0.150
	5	0.200	0.190	0.180
	6	0.250	0.220	0.200
	7	0.320	0.270	0.230
	8	0.400	0.320	0.270
G15R1	0	0.090	-	-
	1	0.110	-	-
	2*	0.140	0.140	-
	3	0.170	0.150	-
	4	0.210	0.160	-
	5	0.270	0.160	-
	6	0.330	0.140	-
	7	0.430	0.130	-
	8	0.480	0.130	-
G18R1	0	0.080	-	-
	1	0.095	-	-
	2	0.120	0.120	-
	3	0.150	0.135	-
	4	0.180	0.140	-
	5	0.210	0.140	-
	6	0.270	0.130	-
	7	0.320	0.120	-
	8	0.400	0.115	-

STRAIN	HOURS POST SUBCULTURE	OPTICAL DENSITY 540nm		
		-M.C.	+ M.C. (0.5 μ g/ml)	+ M.C. (1.0 μ g/ml)
Su298	0	0.080	-	-
	1	0.092	-	-
	2	0.115	-	-
	3*	0.140	0.140	-
	4	0.160	0.150	-
	5	0.190	0.160	-
	6	0.230	0.140	-
	7	0.270	0.130	-
	8	0.350	0.125	-

* = culture split into two or three, equal
volumes and mitomycin added to one, or
two, culture(s) as appropriate.

- = not recorded

Fig.5.3. Effect of Mitomycin on Various Strains.

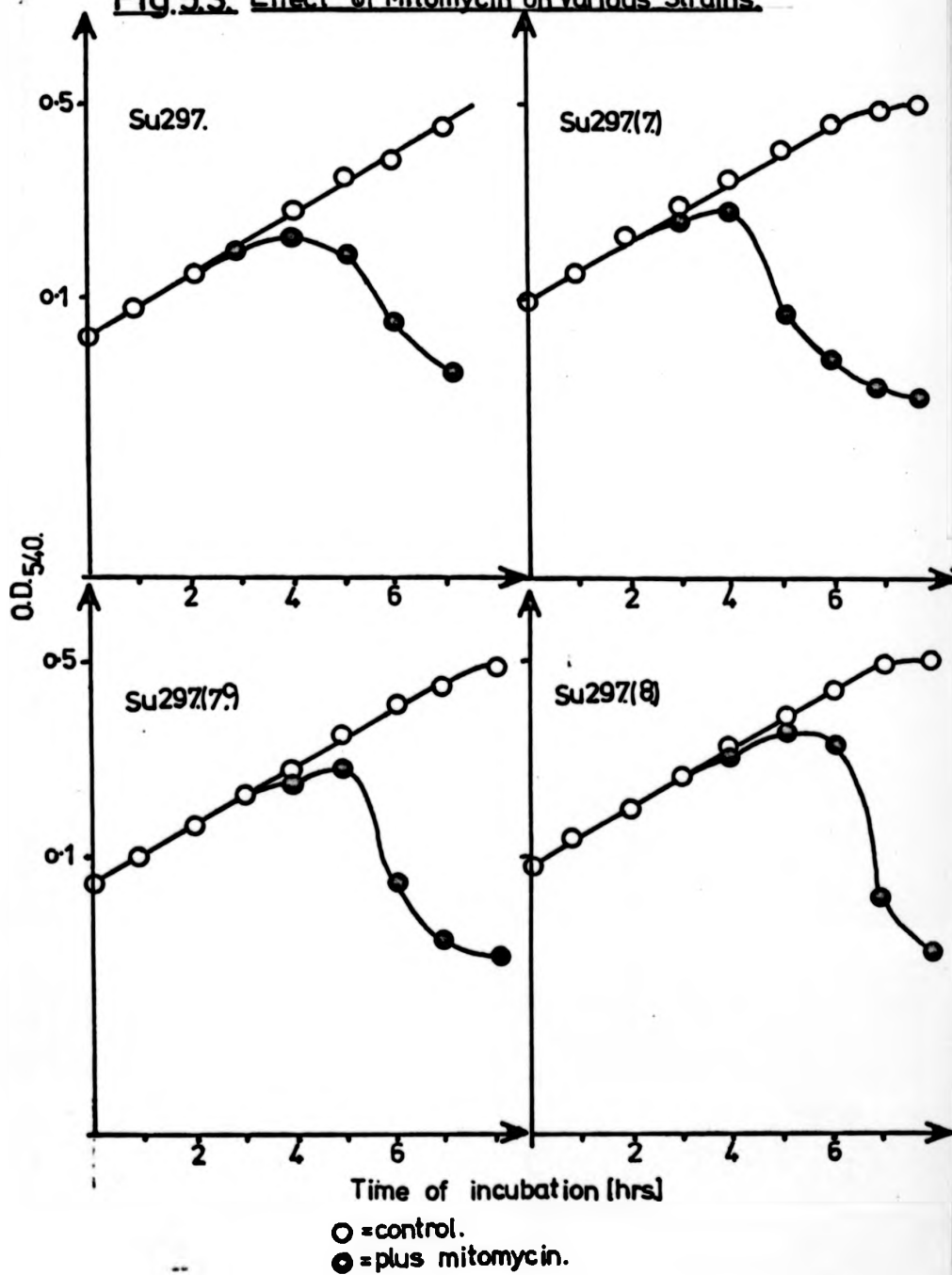


Fig. 5.3.[cont.]

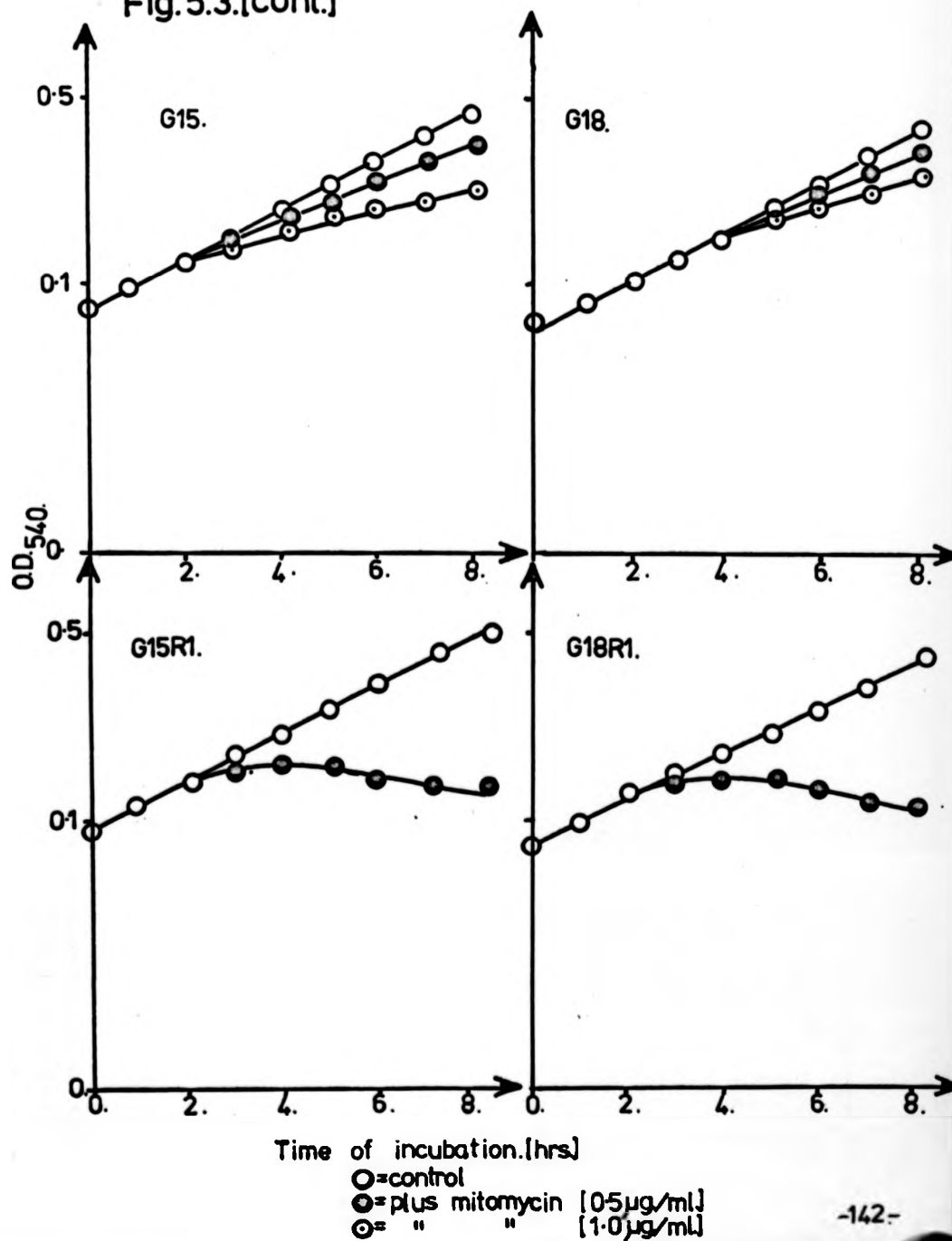
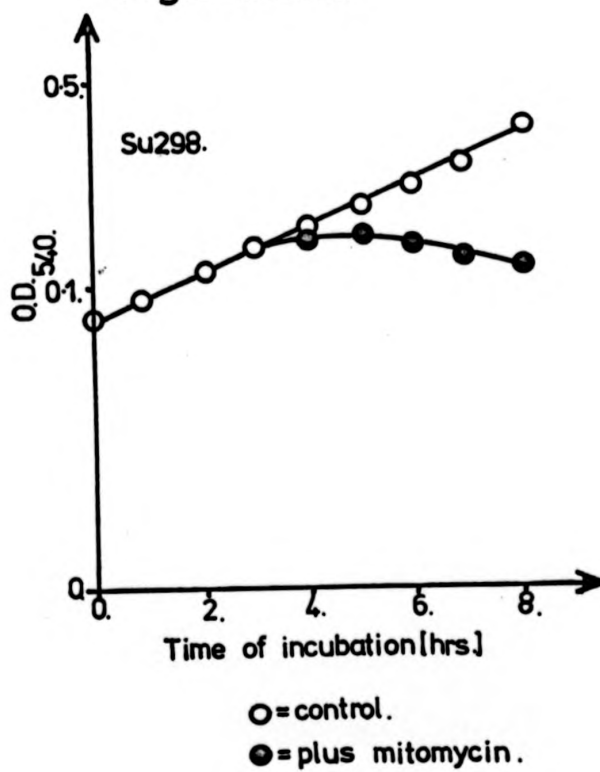


Fig. 5.3[cont]



Discussion:

Kinetics of growth in the presence of mitomycin-C

The results show that MC-sensitivity is a general characteristic of these lysogens. As expected, $\phi 7$, ϕi and $\phi 7^C$ lysogens of Su297 all lyse, on MC treatment, as demonstrated before (Takahashi and Quadling, 1961; Barnet, 1968). Su297(8) reacts in an identical fashion even to the extent of producing clear plaques at a similar frequency to the other phages. From the induction curves Su298 appeared to lyse slightly after MC treatment. Microscopic examination of the MC treated cultures revealed an increased lysis of cells compared with the untreated culture. However, the amount of debris noted was not as much as the lysis debris noted in the other induced lysogen cultures. In fact there was no detectable increase in the extent of lysis in the Su298 cultures compared with that found in the MC-treated G18 and G15 rough mutants. These latter rough mutants had been isolated as ϕC -resistant mutants but they were not lysogenic for any detectable phage. It is interesting that, in culture, these rough mutants are more sensitive to MC than their mucoid parents and this sensitivity was concentration dependent. This finding agrees with the results of the plate assays performed in Chapter Four.

When the Su298 lysates were tested, for phage or bacteriocin release, against a variety of recipients, no activity was detected. Su298 was presumed to be lysogenic for a defective prophage (Takahashi and Quadling, 1961; Barnet, 1968). However, there is no direct evidence in favour of this hypothesis.

Titration of supernatants

From Table 5.14 it can be seen that the MC treated cultures produced considerably more phage than the untreated cultures. In fact the treated cultures released 10^2 - 10^3 more phage than did the untreated cultures. However, although this is a significant stimulation, the final titres of such lysates were not particularly high.

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This is confirmatory evidence of lysogeny since these "lysogens" do carry phage which can be induced by MC treatment.

Table 5.14 Titration of supernatants of MC-treated and control cultures

SUPERNATANT	RECIPIENT HOST	TITRE	PLAQUE TYPE
Su297 + MC	Nu18	3.0×10^7	TURBID*
Su297	"	2.0×10^5	" *
Su297(7)+MC	Su297	1.0×10^8	TURBID*
Su297(7)	"	5.4×10^5	" *
Su297(7 ^C)+MC	Su297	1.5×10^8	CLEAR
Su297(7 ^C)	"	8.5×10^5	"
Su297(8)+MC	Su297	1.1×10^8	TURBID*
Su297(8)	"	6.2×10^5	" *

* = clear plaques arose at 10^{-3} - 10^{-4}

Host range studies

1. Host range of ϕ i

When ϕ i.Su297 was titred on the three hosts, at no time were plaques ever detected on Su297. This is because Su297 is the natural host for ϕ i and so productive infection of Su297 by ϕ i is prevented by the expression of the genes which confer superinfection immunity. However, ϕ i forms plaques on Su298 with an e.o.p. of $\sim 10^{-3}$ of the titre as determined on Nul8. One feature of the plaque formation on Su298 was that there was never any correlation between the dilution factor and the plaque number. In the example in Table 5.15 there were 42 and 34 plaques using the undiluted lysate, yet 6 and 4 plaques on the 10^{-2} dilution plate. The value recorded is an average value and therefore is heavily biased towards the higher level values from the 10^{-2} dilution plates. As mentioned before, the cause of this lack of correlation is unknown although the effect is reproducible.

When ϕ i.Su297.Nul8 was titred the passage of ϕ i through Nul8 reduced the ability of ϕ i to form plaques on Su298 by about 2 logs compared with the value achieved for ϕ i.Su297. Again there was no correlation between the plaque number on Su298 and the dilution factor. Perhaps this effect may be an expression of a random recombinational event between ϕ i and some genetic element in Su298. Even with a titre of 3×10^9 p.f.u./ml. there was no plaque formation on Su297 suggesting that ϕ i^{vir} does not arise at high frequency. Why should Nul8 affect the ability of ϕ i to plaque on Su298? If Su298 is a "restricting" host for ϕ i.Su297 then that could be the reason for the low e.o.p. of ϕ i.Su297 on Su298. The efficiency of the restriction of ϕ i would depend on the state of modification of the ϕ i genome. It may therefore be possible that passage of ϕ i through Nul8 causes a modification of ϕ i such that the phage becomes more susceptible to Su298 restriction.

Table 5.15

Host range of ϕ i

PHAGE	TITRE ON HOST		
	Su297	Su298	Nu18
<u>ϕi.Su297</u> (m.c.i.)	N.D.	$\sim 2.7 \times 10^3$ *	3.0×10^6
<u>e.o.p.</u>	$< 3.3 \times 10^{-6}$	9×10^{-4}	1
<u>ϕi.Su297.Nu18</u>	N.D.	$\sim 5.4 \times 10^4$ *	3.0×10^9
<u>e.o.p.</u>	$< 3 \times 10^{-9}$	1.7×10^{-5}	1
<u>ϕi.Su298</u> (i.e. ϕ i.Su297. Su298)	1.6×10^8	6.8×10^8	1.6×10^8
<u>e.o.p.</u>	2.3×10^{-1}	1	2.3×10^{-1}
<u>ϕi.Su298</u> (i.e. ϕ i.Su297. Nu18.Su298)	4.2×10^8	5.0×10^8	2.5×10^8
<u>e.o.p.</u>	8.4×10^{-1}	1	5.0×10^{-1}
<u>ϕi.Su297. Su298.Nu18</u> (i.e. ϕ 8.Nu18)	N.D.	3.6×10^8	4.9×10^8
<u>e.o.p.</u>	$< 2 \times 10^{-8}$	7.3×10^{-1}	1
<u>ϕi.Su297. Nu18.Su298. Nu18</u> (i.e. ϕ 8.Nu18)	N.D.	3.1×10^8	3.4×10^8
<u>e.o.p.</u>	$< 2.9 \times 10^{-8}$	9.1×10^{-1}	1

* = poor correlation between the titre and the dilution factor (see text)

N.D. = none detected

ϕ i.Su297(m.c.i.) = mitomycin-C induced Su297

Alternatively, passage of ϕ_i through Nul8 could lead to the selection of a mutant phage which adsorbs to Su298 less readily, thereby decreasing the e.o.p. of ϕ_i on Su298 i.e. a host range mutant is selected. Barnet (1968) found that passage of ϕ_7 .Su297 through Nul8 led to the selection of mutant phage which had decreased affinity for the ϕ_7 receptors on Su297. Ultimately, ϕ_7 .Su297.Nul8 was incapable of forming plaques on Su297 at all. In contrast to that progressive effect though, continued passage of ϕ_i through Nul8 did not decrease the ability of the phage to plaque on Su298. Barnet (1968) stated that ϕ_i could not form plaques on Su298. However, she very rarely used lysates with titres higher than 10^6 - 10^7 pfu/ml.

In contrast to Barnet's statement she did refer to an experiment in which she did see plaques on Su298 when she used a higher titre lysate of ϕ_i . Unfortunately, she failed to investigate or characterise these phage plaques further. These experiments show that ϕ_i can indeed plaque on Su298 albeit at low frequency. What phage is responsible for the plaques on Su298? If the plaques on Su298 are due to ϕ_i then they should not be able to plaque on Su297 due to the homoiimmunity of the resident ϕ_i .

Conversely, if the plaques on Su298 are due to a "new" phage which is heteroiimmune with ϕ_i then they may plaque on Su297. According to the models of Takahashi and Quadling (1961) and Barnet (1968) the only phage capable of plating on Su298 is ϕ_8 . This is because, according to these authors, neither ϕ_7 nor ϕ_i can plaque on Su298. The data in table 5.15 show that the phage in the Su298 plaques, when purified on Su298, must be heteroiimmune with ϕ_i because it can form plaques on Su297. Also, this ability to plaque on Su297 is independent of whether or not ϕ_i has been passaged through Nul8 prior to titration on Su298. On the basis of the previous models, this phage ϕ_i .Su298 must really be a new phage, ϕ_8 , formed by ϕ_i -mediated,

specific induction of Su298. Interestingly, if the plaques on Su298 had really been due to ϕ i, one may have expected to find a direct correlation between plaque number and dilution factor. The data also demonstrate that ϕ 8, whether as ϕ i.Su297.Su298 or ϕ i.Su297.Nul8.Su298, plaques about as efficiently on all three hosts. This result contradicts the data of Barnet (1968) according to which there was a reciprocal restriction of ϕ 8, by 10^{-3} , between Su297 and Su298. Also, the plaques of ϕ 8 are large and turbid on Su297 suggesting that ϕ 8 is a temperate phage in Su297. According to the Barnet model for the biogenesis of ϕ 8, ϕ 8 was supposed to be a virulent mutant of ϕ i and so should form clear plaques on Su297. Barnet was unable to isolate Su297(8) lysogens, but in fact they arise with high frequency as found in this study (see page 129.).

Another interesting feature of these data is that passage of ϕ 8 through Nul8 gave rise to a phage which was unable to plaque on Su297. This is curious but is reminiscent of a similar condition noted by Barnet (1968) for ϕ 7. In the case of ϕ 7 the cause was the selection of host range mutants of the phage. It is not impossible that a similar effect is in operation here. Finally, ϕ 8.Nul8 plated as efficiently on Su298 as it did on Nul8, even although it did not plaque on Su297.

2. Host range of ϕ 7

As mentioned before, ϕ 7 can be generated by two methods starting from purified ϕ i.Nul8; namely by broth and plate induction methods. Many experiments were done on the titration of plaque purified ϕ 7 lysates on the three hosts and representative data are presented in Tables 5.16 and 5.17. Although the original ϕ 7 lysate used was different in these two groups of experiments, the data from each passage are approximately the same. It seems likely therefore that the ϕ 7's produced by the plate and broth methods are identical.

Table 5.16

Host range of $\phi 7$ produced by the broth method

PHAGE	TITRE ON HOST		
	Su297	Su298	Nu18
<u>$\phi 7$.Su297</u>	7.3×10^8	4.9×10^2	3.9×10^5
<u>e.o.p.</u>	1	6.7×10^{-7}	5.3×10^{-4}
<u>$\phi 7$.Su297. Su298</u>	1.1×10^8	2.3×10^8	1.6×10^8
<u>e.o.p.</u>	4.8×10^{-1}	1	6.9×10^1
<u>$\phi 7$.Su297. Nu18</u>	N.D.	2.9×10^3	3.9×10^8
<u>e.o.p.</u>	$< 2.6 \times 10^{-8}$	7.4×10^{-6}	1
<u>$\phi 7$.Su297. Su298.Su297</u>	5.2×10^8	4.6×10^1	4.9×10^5
<u>e.o.p.</u>	1	8.8×10^{-8}	9.4×10^{-4}
<u>$\phi 7$.Su297. Nu18.Su298</u>	9.6×10^8	1.2×10^8	9.1×10^8
<u>e.o.p.</u>	8.0×10^{-1}	1	7.6×10^{-1}
<u>$\phi 7$.Su297. Su298.Nu18</u>	N.D.	2.0×10^8	2.2×10^8
<u>e.o.p.</u>	$< 4.5 \times 10^{-8}$	9.1×10^{-1}	1

N.D. = none detected

Table 5.17

Host range of Ø7 produced by the plate method

PHAGE	TITRE ON HOST		
	Su297	Su298	Nu18
<u>Ø7.Su297</u>	4.6×10^8	4.4×10^2	3.1×10^5
<u>e.o.p.</u>	1	9.6×10^{-7}	6.7×10^{-4}
<u>Ø7.Su297.Su298</u>	2.1×10^8	2.5×10^8	1.8×10^8
<u>e.o.p.</u>	8.4×10^{-1}	1	7.2×10^{-1}
<u>Ø7.Su297.Nu18</u>	N.D.	2.0×10^2	1.1×10^7
<u>e.o.p.</u>	$<9.1 \times 10^{-7}$	1.8×10^{-5}	1
<u>Ø7.Su297.Su298.</u> <u>Su297</u>	1.4×10^8	3.0×10^1	2.1×10^5
<u>e.o.p.</u>	1	2.1×10^{-7}	1.5×10^{-3}
<u>Ø7.Su297.Nu18.</u> <u>Su298</u>	2.4×10^7	3.9×10^7	3.2×10^7
<u>e.o.p.</u>	6.2×10^{-1}	1	8.2×10^{-1}
<u>Ø7.Su297.Su298.</u> <u>Nu18</u>	N.D.	3.8×10^7	4.1×10^7
<u>e.o.p.</u>	$<2.4 \times 10^{-7}$	9.3×10^{-1}	1

N.D. = None detected

Table 5.17

Host range of Ø7 produced by the plate method

PHAGE	TITRE ON HOST		
	Su297	Su298	Nu18
<u>Ø7.Su297</u>	4.6×10^8	4.4×10^2	3.1×10^5
<u>e.o.p.</u>	1	9.6×10^{-7}	6.7×10^{-4}
<u>Ø7.Su297.Su298</u>	2.1×10^8	2.5×10^8	1.8×10^8
<u>e.o.p.</u>	8.4×10^{-1}	1	7.2×10^{-1}
<u>Ø7.Su297.Nu18</u>	N.D.	2.0×10^2	1.1×10^7
<u>e.o.p.</u>	$< 9.1 \times 10^{-7}$	1.8×10^{-5}	1
<u>Ø7.Su297.Su298.</u> <u>Su297</u>	1.4×10^8	3.0×10^1	2.1×10^5
<u>e.o.p.</u>	1	2.1×10^{-7}	1.5×10^{-3}
<u>Ø7.Su297.Nu18.</u> <u>Su298</u>	2.4×10^7	3.9×10^7	3.2×10^7
<u>e.o.p.</u>	6.2×10^{-1}	1	8.2×10^{-1}
<u>Ø7.Su297.Su298.</u> <u>Nu18</u>	N.D.	3.8×10^7	4.1×10^7
<u>e.o.p.</u>	$< 2.4 \times 10^{-7}$	9.3×10^{-1}	1

N.D. = None detected

According to Barnet (1968) $\phi 7$.Su297 is restricted to an e.o.p. of 10^{-5} when titred on Nul8. Indeed it can be seen from the data in Table 5.22 that $\phi 7$.Su297 plated less efficiently on Nul8 than on Su297. However, the results here do not show as pronounced an effect as was noted by Barnet i.e. the relative e.o.p.'s are 10^{-3} - 10^{-4} here as compared with 10^{-5} found by Barnet. Plaques arose on Su298 at an e.o.p. of 10^{-6} but because ϕi must necessarily contaminate the lysate it is impossible to say at this stage whether these plaques are due to ϕi or $\phi 7$ infecting Su298. When these plaques were purified and a high titre lysate, made on Su298, was titred on the other hosts, the e.o.p. was approximately as good on all three hosts. This result is similar to that found for the phage produced by ϕi infection of Su298 i.e. $\phi 8$. Again the data mean that the plaques formed on Su298 by the $\phi 7$.Su297 lysate are due to a phage heteroimmune with ϕi . Hence these plaques on Su298 could be due to either $\phi 8$, $\phi 7$ or the formation of a novel phage caused by the interaction of $\phi 7$ with Su298. Unfortunately, it is impossible to test for homoimmunity either with $\phi 7$ or $\phi 8$, against Su297(7) or Su297(8) as these lysogens are subject to complete cross resistance due to phage conversion (Barnet, 1968; this study).

When $\phi 7$.Su297 was passaged through Nul8 and titred on the other hosts, an interesting phenomenon occurred. The resultant $\phi 7$.Nul8 was no longer capable of plating on Su297 although plaques did form on Su298 with an e.o.p. of 10^{-5} . Barnet (1968) noted this effect and attributed it to the selection of host range mutants of $\phi 7$ on Nul8. With each passage of $\phi 7$ through Nul8 the proportion of phage in the lysate which was capable of adsorbing to Su297 decreased. Consequently, by the third cycle of growth of $\phi 7$ on Nul8, there were no phage particles capable of adsorbing to Su297. However, Barnet also stated that $\phi 7$.Nul8 was subject to restriction by Su297 such that $\phi 7$.Nul8 plated on Su297 at an e.o.p. of 10^{-5} . Barnet's data for

restriction was derived from a $\phi 7$.Nul8 lysate which had been passaged through Nul8 only once from Su297. Obviously, if both effects were present it would be difficult to differentiate between them. Barnet by no means proved that restriction of $\phi 7$ does operate between Su297 and Nul8 because her data were derived from single cycle lysates and so the presence of ϕi could easily have complicated the results. When $\phi 7$.Su297 is titred on Nul8, the plaques which arise could be due to either ϕi or $\phi 7$. These plaques could have been due to ϕi if Nul8 was $\phi 7$ resistant or if Nul8 restricted $\phi 7$ with great efficiency. If these plaques were due to ϕi then the lysate, when purified, would not cause plaque formation on Su297 due to the resident ϕi immunity. Consequently, this model is equally as plausible as the restriction and host range mutant selection model of Barnet (1968). From the same experiment $\phi 7$.Su297.Nul8 formed plaques on Su298 with an e.o.p. of 10^{-5} . Again, these plaques could be due to either $\phi 7$ or ϕi . Previous data (see Table 5.15) has shown that ϕi .Nul8 plates on Su298 with an e.o.p. of 10^{-5} . Hence, if the plaques of $\phi 7$.Su297.Nul8 on Su298 are due to $\phi 7$ then they arise with the same frequency as was found for the ϕi .Nul8 lysate.

Although $\phi 7$.Su297.Su298 can plate as efficiently on all three hosts, subsequent passage of this phage through Su297 resulted in a phage which plated very inefficiently on Su298 (10^{-7}) and Nul8 (10^{-3}). This phage, $\phi 7$.Su297.Su298.Su297 could be either $\phi 7$ or $\phi 8$ according to the same arguments of previous logic. Whatever the identity of the phage it means that Su297 somehow "modifies" the phage such that its ability to plate on Su298 and Nul8 is reduced i.e. as though Su298 were a restricting host and Su297 were a modifying host.

When $\phi 7$.Su297 is passaged through Nul8 before passage through Su298 it again leads to a phage which plated efficiently on all three hosts. However, passage of $\phi 7$.Su297.Su298 through Nul8 leads to the

production of a phage which cannot plate on Su297. If the phage $\phi 7.Su297.Su298$ is really due to $\phi 8$ then it appears that $\phi 8$ is subject to the same inability to plate on Su297, as $\phi 7$, when it is passaged through Nul8.

An interesting fact is that when one compares the e.o.p., on Su298, of $\phi 7.Su297.Nul8$ with $\phi 7.Su297.Su298.Nul8$ they plate with different efficiencies. Although $\phi 7.Su297.Nul8$ appears to be "restricted" by Su298 the effect is less pronounced than the "restriction" of $\phi 7.Su297$ by Su298. So passage of $\phi 7$ via Su298 prior to passage through Nul8 has somehow "trained" $\phi 7$ to plaque on Su298. It must always be borne in mind in this analysis that passage of any of these phages through Su297 or Su298 may lead to the generation of a novel phage due to the lysogenic status of the latter two strains. Consequently, the analysis of host range in this system is subject to these possibilities and must therefore be speculative. This fact is even more noticeable when one considers the original lysate for all of these studies i.e. the lysate designated $\phi 7.Su297$. By definition this phage is really $\phi i.Su297.Nul8.Su298.Su297$. Such studies exemplify and reinforce the great need for comparative molecular studies on purified phage.

3. Host range of Ø8

As with Ø7 the data in Tables 5.18 and 5.19 show that similar results were obtained whether the original Ø8 was produced by broth or plate induction methods. Therefore the same phage is produced by either method. The data show that Ø8.Su298 plates as efficiently on all three hosts. Consequently, neither Su297 nor Nul8 restrict Ø8. This contradicts the claim that Ø8 is restricted by Su297 (Barnet, 1968). When Ø8.Su298.Su297 was titred on the three hosts no plaques were noted on Su298, although this particular titration was repeated three times. This is a remarkable result since, even if Ø8 cannot plate on Su298 under these circumstances, it may be expected that the contaminating Øi would form plaques on Su298 by the specific induction of Ø8. Perhaps all of the Øi adsorption sites on Su298 were "occupied" by Ø8 which, for some reason, cannot replicate in Su298 after passage through Su297. This lysate, Ø8.Su298.Su297, can form plaques on Nul8 but at a reduced e.o.p. i.e. 10^{-3} . This is the same e.o.p. as was achieved for Ø7.Su297 when titred on Nul8. This is interesting and may be circumstantial evidence in favour of the hypothesis that the plaques on Nul8 are not due to Ø8 but Øi.

The data for the titration of Ø8.Su298.Nul8 reveal several interesting features. As with Ø7.Nul8, Ø8.Su298. Nul8 is incapable of plating on Su297. The reason for this result is obscure but, in contrast to the result with Ø7 it is impossible that the Ø8.Su298.Nul8 lysate could really be Øi. Hence the inability of Ø8.Su298.Nul8 to plaque on Su297 must be a direct effect on Ø8, and it must be manifested only by its passage through Nul8. It is not possible for the Ø8.Su298.Nul8 lysate to be anything else but Ø8 since Ø8.Su298 plated on Nul8 as effectively as it did on the other two hosts. Hence, just as Barnet (1968) found for Ø7, Nul8 selects out derivatives of Ø8

Table 5.18

Host range of $\phi 8$ produced by the broth method

PHAGE	TITRE ON HOST		
	Su297	Su298	Nul8
<u>$\phi 8$.Su298</u>	3.3×10^8	4.2×10^8	1.9×10^8
<u>e.o.p.</u>	7.8×10^{-1}	1	4.5×10^{-1}
<u>$\phi 8$.Su298. Su297</u>	1.3×10^8	N.D.	2.0×10^5
<u>e.o.p.</u>	1	$< 7.7 \times 10^{-8}$	1.5×10^{-3}
<u>$\phi 8$.Su298. Nul8</u>	N.D.	7.1×10^8	6.9×10^8
<u>e.o.p.</u>	$< 1.4 \times 10^{-8}$	1	1
<u>$\phi 8$.Su298. Nul8.Su298</u>	5.9×10^8	8.2×10^8	6.7×10^8
<u>e.o.p.</u>	7.2×10^{-1}	1	8.2×10^{-1}
<u>$\phi 8$.Su298. Su297.Nul8</u>	N.D.	1.0×10^3	5.0×10^7
<u>e.o.p.</u>	$< 2.0 \times 10^{-7}$	2.0×10^{-5}	1

N.D. = none detected

Table 5.19 Host range of Ø8 produced by the plate method

PHAGE	TITRE ON HOST		
	Su297	Su298	Nu18
<u>Ø8.Su298</u>	7.0×10^7	7.9×10^7	6.8×10^7
<u>e.o.p.</u>	8.9×10^{-1}	1	8.7×10^{-1}
<u>Ø8.Su298.</u> <u>Su297</u>	1.8×10^8	N.D.	2.1×10^5
<u>e.o.p.</u>	1	$< 5.5 \times 10^{-8}$	1.2×10^{-3}
<u>Ø8.Su298.</u> <u>Nu18</u>	N.D.	3.6×10^8	4.3×10^8
<u>e.o.p.</u>	$< 2.3 \times 10^{-8}$	8.4×10^{-1}	1
<u>Ø8.Su298.</u> <u>Nu18.Su298</u>	8.9×10^7	1.1×10^8	9.2×10^7
<u>e.o.p.</u>	8.1×10^{-1}	1	8.4×10^{-1}
<u>Ø8.Su298.</u> <u>Su297.Nu18</u>	N.D.	5.35×10^4	6.6×10^8
<u>e.o.p.</u>	$< 1.5 \times 10^{-8}$	8.3×10^5	1

N.D. = None detected

which are incapable of plating on Su297. No adsorption tests were done on any of these phage/host studies and so it is impossible to say at this stage if the reason for this effect is the same in both Ø8 and Ø7. Although Ø8.Su298.Nul8 cannot plate on Su297 it plated as efficiently on Su298 as it did on Nul8. Hence this "adsorption effect" (Barnet, 1968) is highly strain specific.

When Ø8.Su298.Nul8.Su298 was purified and titred on the three hosts it had regained the host range and e.o.p. of the original Ø8.Su298. Therefore, passage through Nul8 did not lead to a heritable change in the ability of Ø8.Su298 to plate on all hosts. This also means that, although Ø8.Su298.Nul8 cannot plate on Su297, passage of this phage through Su298 eliminates this inability to plaque on Su297.

Finally, when Ø8.Su298.Su297.Nul8 was titred on the three hosts it was incapable of plating on Su297. However it plated on Su298 with an e.o.p. of 10^{-5} . If the growth on Nul8 had been due to the selection of Øi from the lysate it would have been expected that the lysate raised on Nul8 would have plated on Su298 with an e.o.p. of 10^{-5} - 10^{-6} , as Øi.Nul8 did. This could be evidence in favour of the hypothesis that Nul8 does first select Øi from lysates of Ø7 or Ø8 grown on Su297. In other words Øi.Su297 is not restricted by Nul8 either.

The basic relationships between Øi, Ø7, Ø8 and Su297, Su298 and Nul8 are summarily depicted in Figure 5.4.

4. Host range of Ø7^C

The data (Table 5.20) show that when Ø7^C.Su297 was titred on all hosts it plated with low efficiency on Su298 (10^{-7}) and Nul8 (10^{-5}). However, Ø7.Su297 plated on Nul8 with an e.o.p. of 10^{-3} and the plaques produced were turbid (see ~~pages 19-21~~). The low e.o.p. of Ø7^C.Su297 on Nul8 could be due to the fact that Nul8 may be Ø7 resistant and so the low plaque numbers may be due to contaminating Øi. Alternatively, Ø7^C.Su297 may be "restricted" by Nul8 and it

may simultaneously fail to express the clear plaque phenotype, as expressed on Su297. The $\phi 7^C$.Su297 lysate plated as efficiently as $\phi 7$.Su297 did on Su297 but it was difficult to tell if the plaques were clearer in the case of $\phi 7^C$. The $\phi 7^C$.Su297.Nul8 lysate was incapable of forming plaques on Su297, as was $\phi 7$.Nul8 (see earlier). However, such a lysate ($\phi 7$.Su297.Nul8) could plaque on Su298 with an e.o.p. of 10^{-5} . Again, this frequency is of the order of the e.o.p. frequency obtained for ϕi .Su297.Nul8 on Su298.

Table 5.20Host range of Ø7C.

PHAGE	TITRE ON HOST		
	Su297	Su298	Nu18
<u>Ø7^C.Su297</u>	9.2×10^8	2.8×10^2	$7.7 \times 10^3^*$
<u>e.o.p.</u>	1	3.0×10^{-7}	8.4×10^{-6}
<u>Ø7^C.Su297.</u> <u>Nu18</u>	N.D.	2.9×10^3	4.2×10^8
<u>e.o.p.</u>	$< 2.4 \times 10^{-8}$	6.9×10^{-6}	1

N.D. = None detected

* = turbid plaques rather than clear plaques.

Summary of the host range data

According to Barnet (1968), $\phi 7$ was restricted by Su297 and Nul8 because the phage plated with low efficiency (10^{-5}) on one host after being grown on the other. The conclusion that restriction and modification operates in Su297 and Nul8 can be criticised for the following reasons. From the data of Barnet (1968) and this study, $\phi 7$.Nul8 cannot plate on Su297 after three cycles of growth on Nul8. Barnet ascribed this fact to the discovery that growth of $\phi 7$.Su297 on Nul8 led to the selection of host range mutants of $\phi 7$ which could adsorb to Nul8 but not Su297. Because of this effect, Barnet was forced to derive her data of the e.o.p. of $\phi 7$.Nul8 on Su297 using lysates of $\phi 7$.Nul8 which had been made from $\phi 7$ passed through Nul8 only once. It is not impossible that such a lysate could have been contaminated by ϕi from Su297. Hence it was not conclusively proved by the study of Barnet, or by this study, that the lysate called $\phi 7$.Nul8 was not really just ϕi . If $\phi 7$ is incapable of plaquing on Nul8 then growth of a $\phi 7$.Su297 lysate on Nul8 would select contaminating ϕi from the lysate and therefore yield an e.o.p. of reduced frequency on Nul8. Hence the low e.o.p. of $\phi 7$.Su297 (of 10^{-5} in Barnet's study and 10^{-3} here) on Nul8 may not necessarily be due to restriction. By the same argument, the low e.o.p. 's may not even be due to the selection of host range mutants, which can adsorb better to Nul8, but may be due to the selection of "contaminant" ϕi from the lysate. Growth of 1 cycle " $\phi 7$ ".Nul8 on Su297 could, correspondingly, be ascribed to residual $\phi 7$ carried over into what would really be the ϕi .Nul8 lysate. There is insufficient evidence in this study to determine which, if any, of these possibilities is correct. Also, in Barnet's work, Nul8 "restricted" $\phi 7$ by 5 logs but this study shows that the effect is not so pronounced (3 logs). The reason for this difference is unknown.

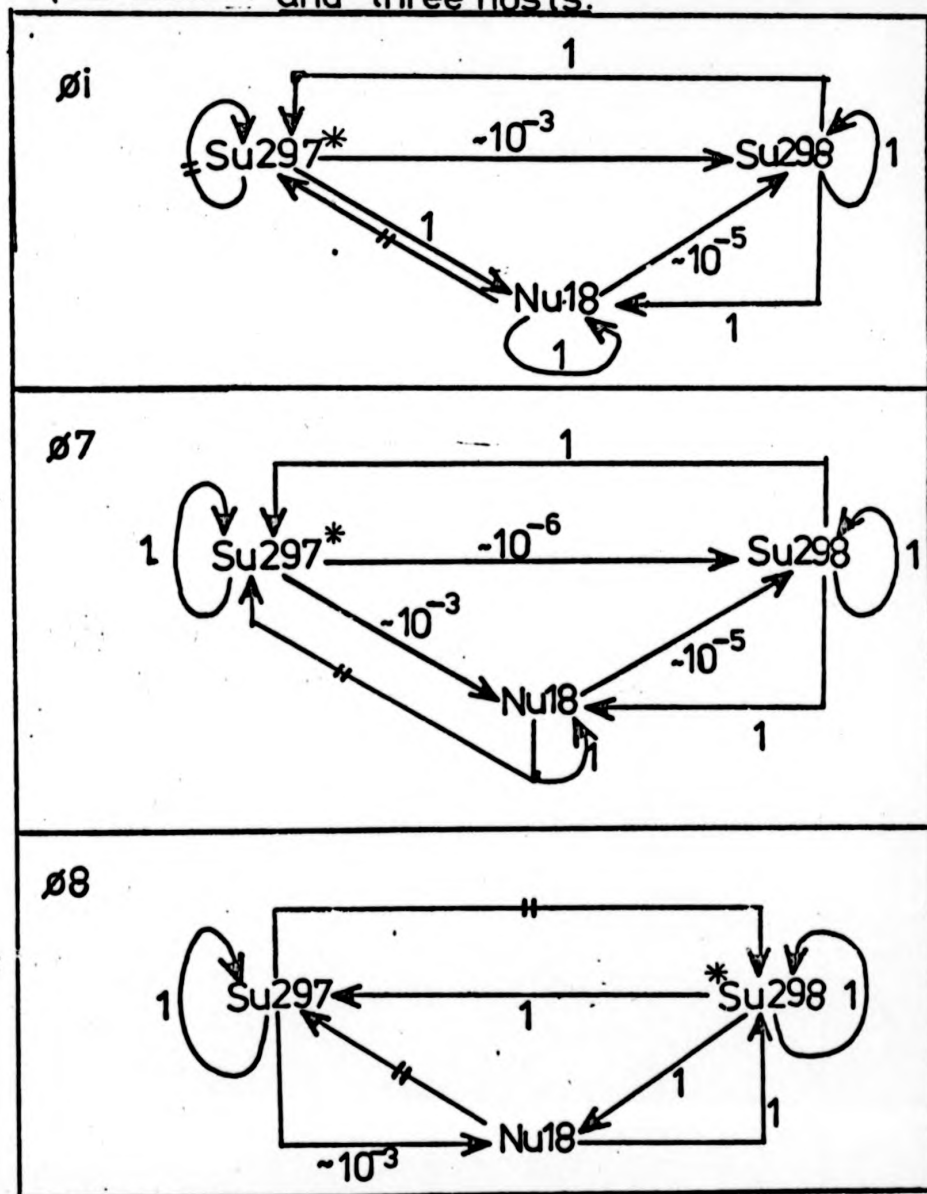
According to Barnet (1968) Ø8 was subject to restriction and modification, by Su297 and Su298, by 3 logs. Although this study shows that Ø8.Su297 plates with low efficiency, if at all, on Su298, Ø8.Su298 plates with about equal efficiency on all three hosts. This discrepancy between the data of Barnet and the data found in this study cannot be explained without further experiment. Overall, however, Barnet's claim for a restriction and modification system operating between Su297, Su298 and Nul8 are not completely justified by the findings of this study.

Barnet also claimed that adsorption mutants of Ø7 were selected when Ø7.Su297 was titred on Nul8. However she could not exclude the possibility that she had selected out Øi from the Su297 lysate and that either Ø7 could not plate on Nul8 at all or, if it could, at a reduced e.o.p. The data from this study show that an identical effect can be found for Ø7 and Ø8 when grown on Nul8 but there are insufficient data to differentiate between the two possibilities of adsorption mutant selection or Øi selection. However, when Ø8.Su298 was grown on Nul8 it plated with an e.o.p. of 1. Hence the phage lysate produced on Nul8 must have been Ø8. Such a Ø8.Nul8 lysate was incapable of plating on Su297 and this makes it seem likely that growth on Nul8 selects phages which have altered adsorption rates, to Su297. It is interesting in this respect too that, although Øi.Su297 plates on Su298 with an e.o.p. of 10^{-3} , Øi.Nul8 plates on Su298 with an e.o.p. of 10^{-5} . Therefore, again, passage of Øi through Nul8 decreased its ability to plate on Su298.

Finally, Barnet stated that the appearance of the phage plaque type was host dependent e.g. Ø7^C.Su297 gave clear plaques on Su297 but turbid plaques on Nul8. The data in this study show that such effects can be shown to be repeatable. However it cannot be certain that, if Nul8 is Ø7^C resistant, that Øi was selected by Nul8 from

the Su297-grown lysate. Indeed, $\phi 7^C$.Su297.Nul8 reacts exactly as ϕi .Nul8 reacts in terms of host range. Hence the fact that turbid plaques are formed by $\phi 7^C$.Su297 on Nul8 can equally well be presented as evidence in favour of the hypothesis that Nul8 selects ϕi from the lysate rather than the idea that $\phi 7^C$ naturally forms turbid plaques on Nul8.

Fig. 5.4. Basic relationships between ϕ_i, ϕ_7, ϕ_8 and three hosts.



*=original source of phage

Phage characterisation on one host

Ultraviolet light inactivation of plaque forming ability

The previous host range studies and studies on the biogenesis of $\phi 7$ and $\phi 8$ show that $\phi 7$, $\phi 8$ and ϕi are very closely related. In an attempt to characterise these phages better they were subjected to thermal and UV inactivation. Barnet (1968) had previously attempted this and stated that these phages could be differentiated by UV inactivation kinetics i.e. $\phi 8$ was more closely related to ϕi than $\phi 7$. In fact this similarity in the inactivation rates of ϕi and $\phi 8$ was one of the few features which led Barnet to propose the model in which $\phi 8$ was a virulent mutant of ϕi . However, Barnet did concede that there was significant variation between experiments in UV inactivation; serology and single step growth curve studies, which she could not explain. It was important for this reason to test the repeatability of these UV inactivation experiments. For the results to be significant, the phages all have to be treated under identical conditions and this means growing the phages on the same host and plating the inactivated phages on the same host. This host was Nul8 and the results are presented in Figures 5.5 and 5.6.

Figure 5.5 shows the inactivation of ϕi , $\phi 7$ and $\phi 8$ by UV, as titred on Nul8. It also shows the inactivation of $\phi S28$ and ϕi as titred on Gl8. Because different hosts were used to titre the phages these data only suggest that ϕi , $\phi 7$ and $\phi 8$ are smaller phages than $\phi S28$ and ϕC . There was some variation in the plaque numbers in these experiments and this may have contributed to the departure from linearity of these inactivation rates. From this experiment $\phi 7$, ϕi and $\phi 8$ react similarly. If $\phi 8$ is related to either $\phi 7$ or ϕi it would appear to be more closely related, in terms of genome size, to $\phi 7$ rather than to ϕi . However, these data are not adequate for the differentiation of these phages on the ground of UV inactivation. The data represented in Figure 5,5

Fig. 5.5 U.V. inactivation of $\phi i, \phi 7, \phi 8$ & $\phi S28$.

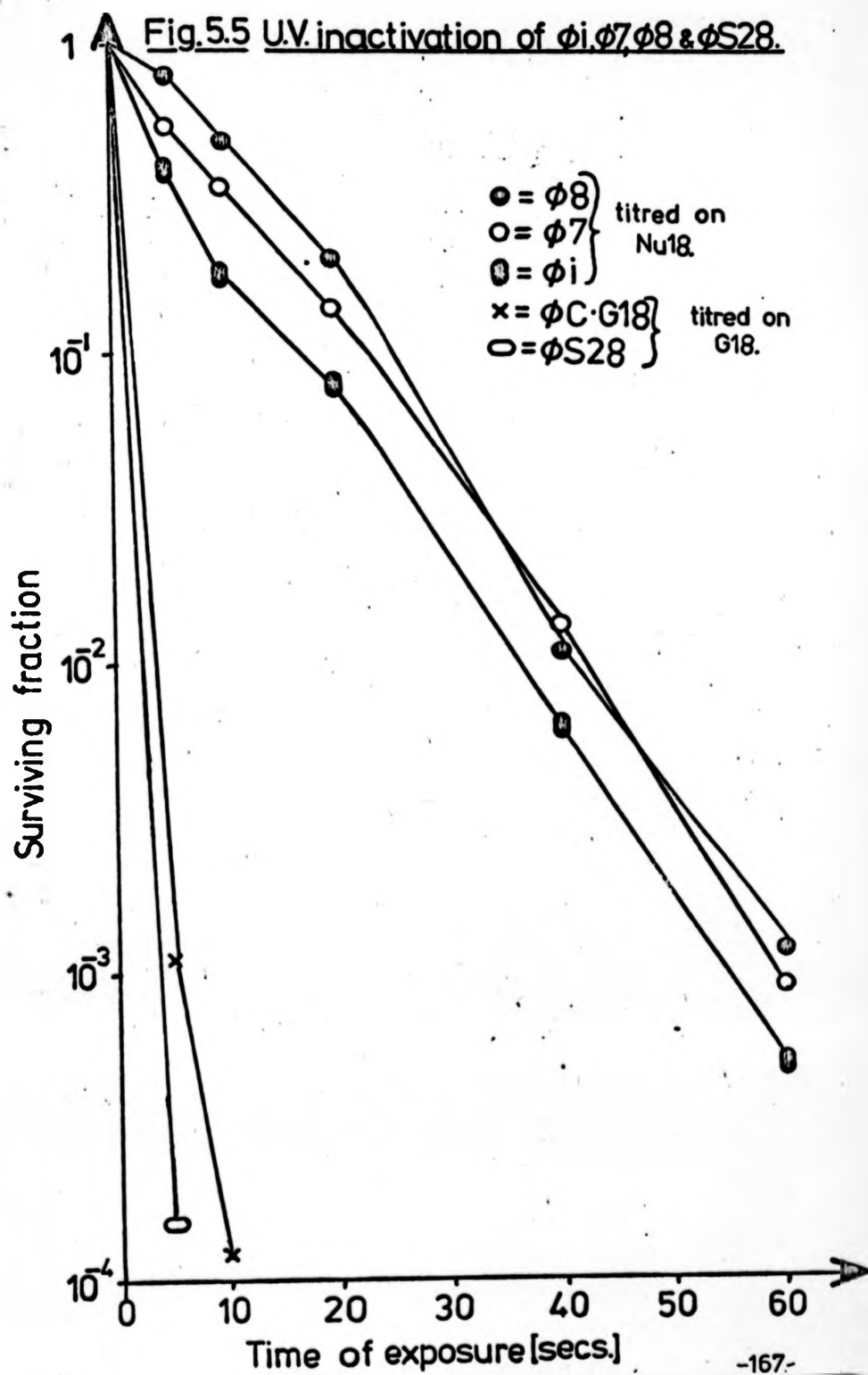


Fig. 5.6 U.V. inactivation of ϕ i, ϕ 7 & 8.

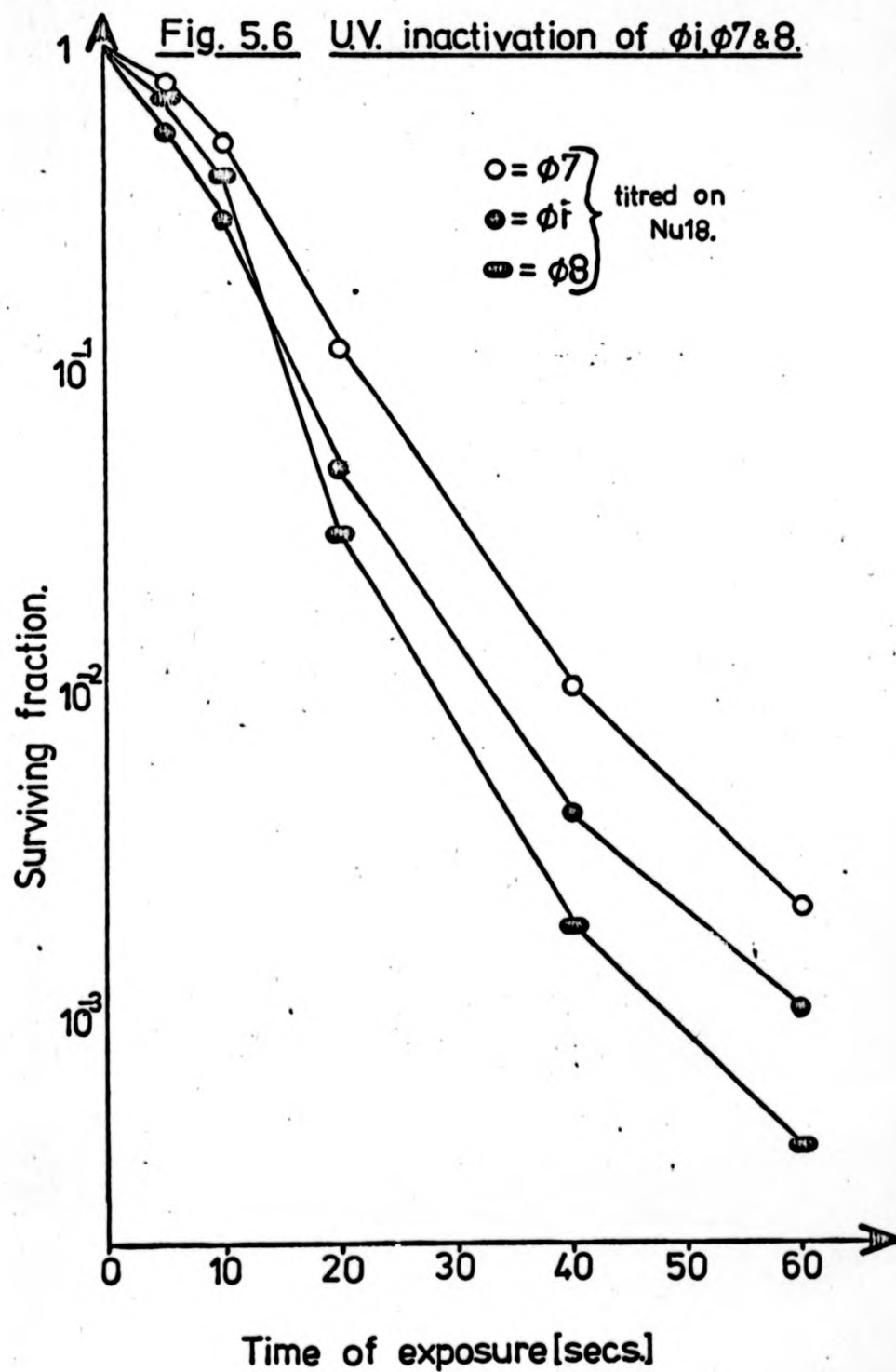
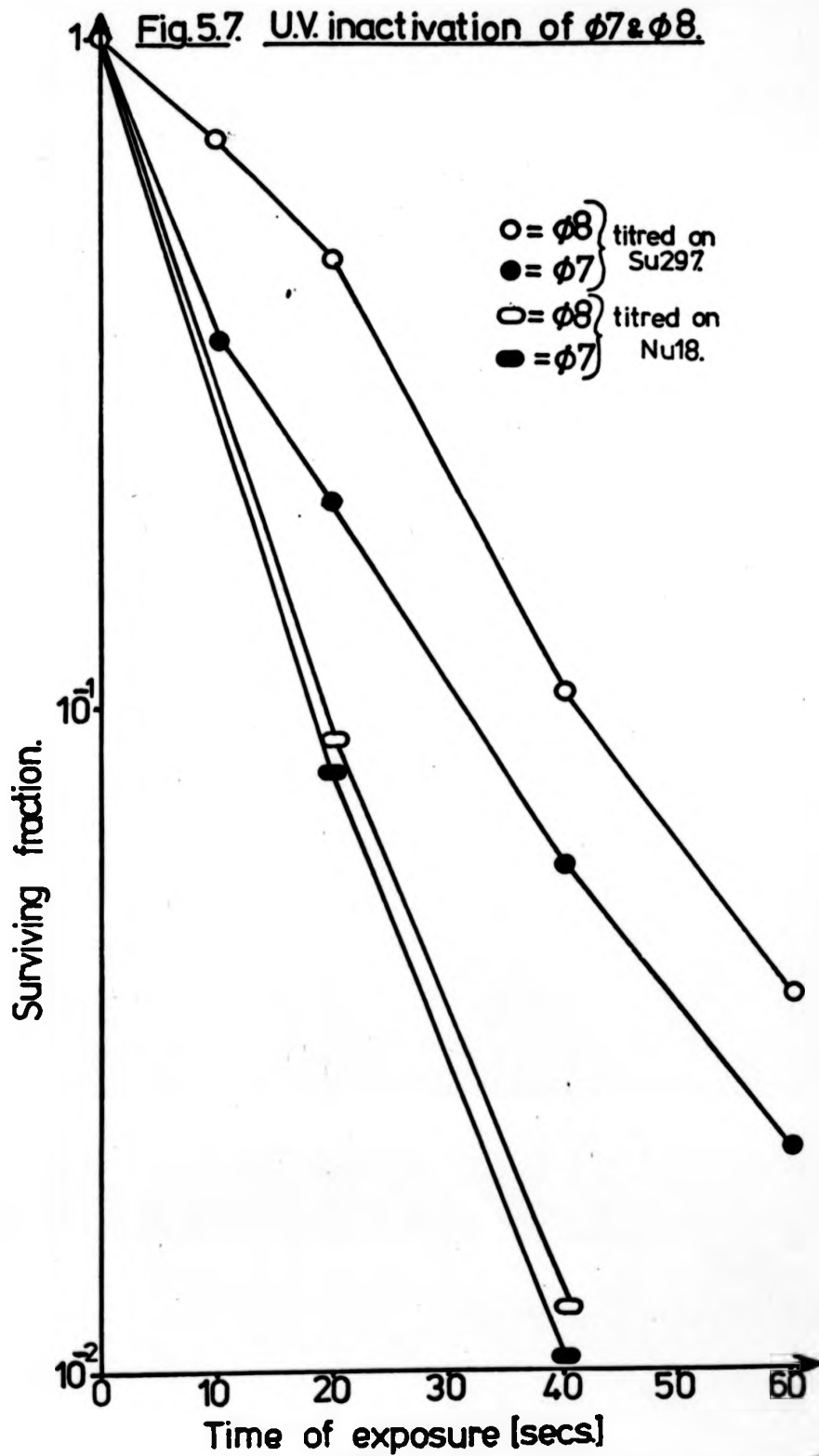


Fig.5.7 U.V.inactivation of $\phi 7$ & $\phi 8$.



reinforces this conclusion. None of these three curves shows linearity and, in total contrast to the previous result, Ø1 shows slightly greater resemblance to Ø8 than Ø7 in its inactivation rate. The reason why these curves are not linear is unknown. Perhaps the rate of inactivation is affected by debris present in the diluted lysate.

At this stage all that can be concluded from these experiments is that the three phages are closely related in the size of their genome. Because there is variation from one experiment to another and because there is not a significant difference in the inactivation rates, it is impossible to differentiate these phages on the grounds of UV inactivation kinetics.

The data in Figure 5.7 show that after 50-60 seconds treatment Ø7 and Ø8 had only decreased in titre by $1\frac{1}{2}$ logs when titred on Su297. This is a significant difference from the rate of inactivation of both phages as titred on Nul8. There could be several reasons for this. Su297 may "repair" UV-induced damaged phage more efficiently than Nul8 can. Alternatively, the amount of interfering debris in the lysate produced on Su297 could be greater than that in the Nul8 lysates. The reason for this difference was unknown and was not investigated further.

From the thermal inactivation curves (Figure 5.8) all three phages show a very rapid inactivation of plaque forming ability. Even though their original titres varied, each lysate lost $2\frac{1}{2}$ -3 logs activity after only five seconds. However, after this initial dramatic decrease in activity there was very little decrease in titre on continued heat exposure. Why should the thermal inactivation curves show such a dramatic biphasic response? Barnet and Vincent (1970) suggested that anomalies in the UV and thermal inactivation curves of Ø7 could be due to the reversible inhibition

of Ø7 by adsorption to host debris. According to their report these effects could be reversed by using L.S.B. as the medium in which the lysate was generated. However when this was tested no obvious difference in the thermal inactivation curves was noted (data not presented). Presumably, according to Barnet and Vincent, the rapid inactivation phase could be due to the inactivation of free phage in the lysate whereas the subsequent slow phase of inactivation could be due to the inactivation of debris-protected phage.

Whatever the reason for the biphasic kinetics of inactivation, it becomes clear that these phages cannot be differentiated by the criterion of thermal inactivation kinetics. It must be concluded, therefore, that the differences between these three phages are so small that such crude methods as UV and thermal inactivation are totally inadequate as methods for the differentiation of one phage from another. Hence methods which are more sensitive to very slight differences in phage nucleic acid and coat proteins need to be employed here. Such methods include electron microscopy to examine and compare the phages morphologically as well as to examine heteroduplexes of cross-hybridised nucleic acid. Alternative methods of analysis would be to examine the coat proteins of each phage and determine the molecular weights of the phage genomes. To do such analyses high titres of phage are required and, to be strictly comparative, they must all be grown on the same host, Nul8.

Finally, comparative restriction enzyme analysis of the DNA from the three phages revealed:

- 1) The DNA from all three phages co-migrates.
- 2) There are no Eco RI sites in any of these phages DNA.
- 3) The molecular weight of the DNA of these phages is $1 - 2 \times 10^7$ and thus cannot have a large coding capacity.

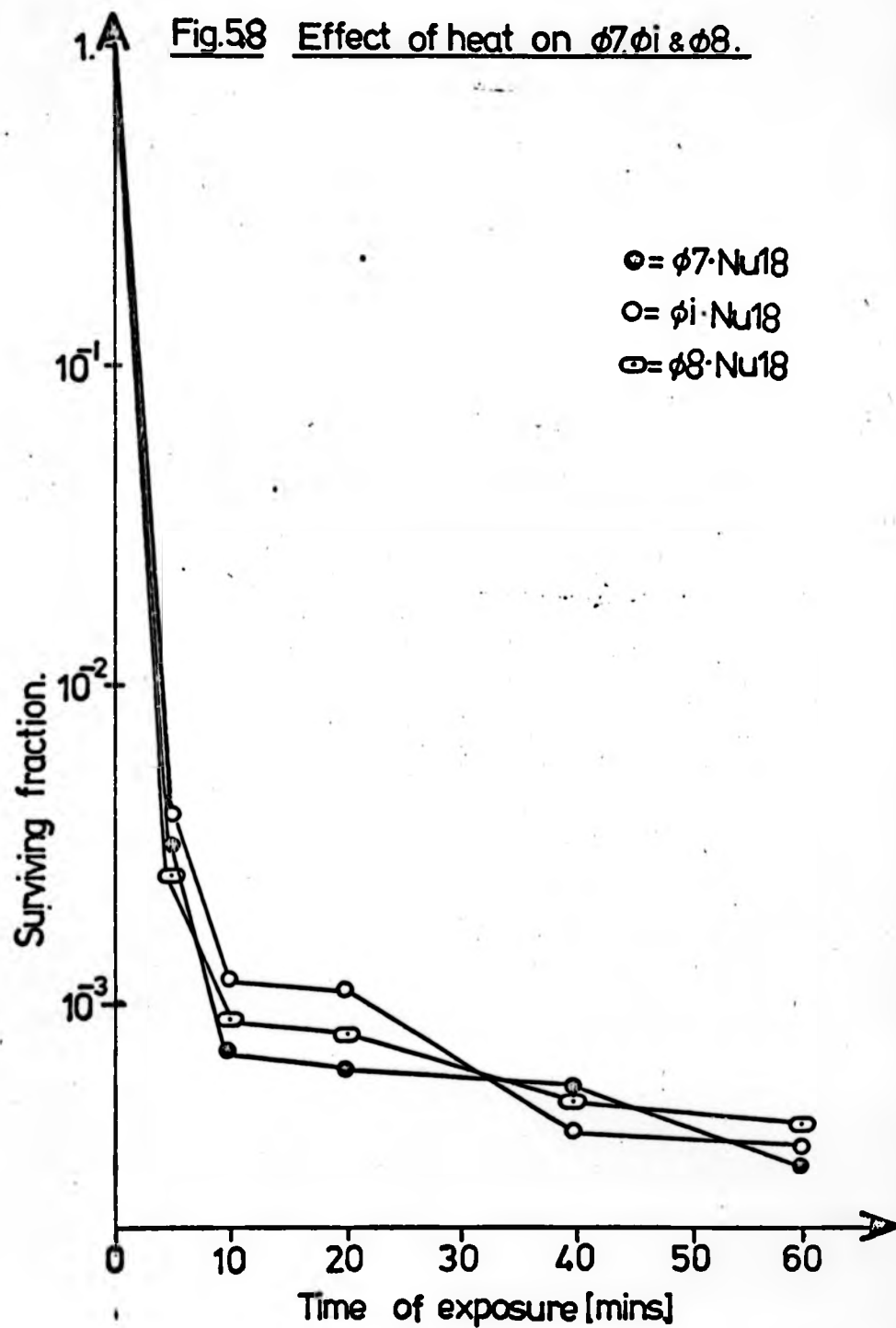
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Fig.58 Effect of heat on $\phi 7$ & $\phi 8$.



The production of high titre lysates

A. Broth methods

1) Mitomycin C induction of lysogens

As mentioned earlier, MC stimulated phage release from $\phi 7$, $\phi 8$ and ϕi lysogens of Su297 but the stimulation in titre, although by about 2 logs, never led to a final titre of much greater than 10^9 pfu/ml. Barnet (1968) found that ϕi and $\phi 7$ were released from the host with a burst size of 30-40 in Nul8. Consequently, from induction of a log phase culture of a lysogen, one would expect to produce more than 10^9 pfu/ml. Therefore, the yields achieved here were low. Perhaps adsorption of some phage to debris was a problem, although MC induction in LSB did not increase the phage recovery to any significant extent contrary to the suggestion of Barnet and Vincent (1970).

2) Broth infection of log phase Nul8

When log phase Nul8 cultures were infected at m.o.i's of 0.1 to 2 the lysates produced yielded no higher titres than those produced by MC induction of lysogens. This method had the added disadvantage of requiring high titres of phage as the input lysate.

B. Plate methods

Using the plate methods described in the materials and methods, higher titre lysates were achieved. However, the titres were variable. Using identical methods with ϕC and $\phi PRR1$ titres of 10^9 - 10^{11} pfu/ml. were achieved. These methods proved equally successful for the production of high titre lysates of some coliphages (N. Seeley, pers.comm.). Unfortunately, the titres of ϕi , $\phi 7$ and $\phi 8$ lysates produced by these methods were never as high as for the other phages mentioned above. On rare occasions, titres of over 10^9 pfu/ml. were achieved but the vast majority of lysates produced contained 10^7 - 10^9 pfu/ml. Another problem with many of

these lysates produced on Nul8 was the mucoid condition of the final lysate, due to the copious extra-cellular polysaccharide produced by the host.

It seems likely then that the low recovery of phage in these experiments was not a reflection of any inadequacies in the technology used since recovery of other phages was high. This may mean that the low recovery is a reflection of some feature unique to the phage/host interaction in this system.

Concentration of phage from lysates

Because high titre lysates could not be achieved using the methods described in the previous section, the only way left to increase the phage titres was to try to concentrate the phages into a smaller volume. Four techniques were used in the concentration and purification of these phages namely, polyethylene glycol 6000 precipitation (peg precipitation) (Yamamoto *et.al.*, 1970); density gradient centrifugation; dialysis and, finally, diaflo ultra-filtration. Using the first three of these four methods three phage lysates, Ø1, Ø7 and Ø8 were concentrated. Samples were removed at each stage for titration to determine the efficiency of recovery after each treatment. Lysates produced from top agar layers were titred after thirty minutes contact with the top agar. Also, these lysates from macerated top agar were left at 4°C overnight to enhance the elution of phage into the supernatants. At this stage the samples were titred before these supernatants were used for peg precipitation. The results are presented in Table 5.21 and represented in Figure 5.9.

Figure 5.9 shows that, for all three phage lysates, overnight elution of phage at 4°C enhanced the titre by 150-300%. It is not known whether or not prolonged treatment at 4°C would increase the recovery. The peg precipitation appears to be efficient because the recovery of each phage was boosted considerably by this treatment. Samples of 100 ml. lysates were concentrated down to 5 ml. after peg concentration and so recovery from such treatments must be about 100%. The most pronounced result of these experiments, however, was that after CsCl density gradient equilibrium centrifugation and dialysis, the titre of the phage recovered decreased dramatically. In fact about 3 logs of activity were lost between the pre-centrifugation and post-dialysis

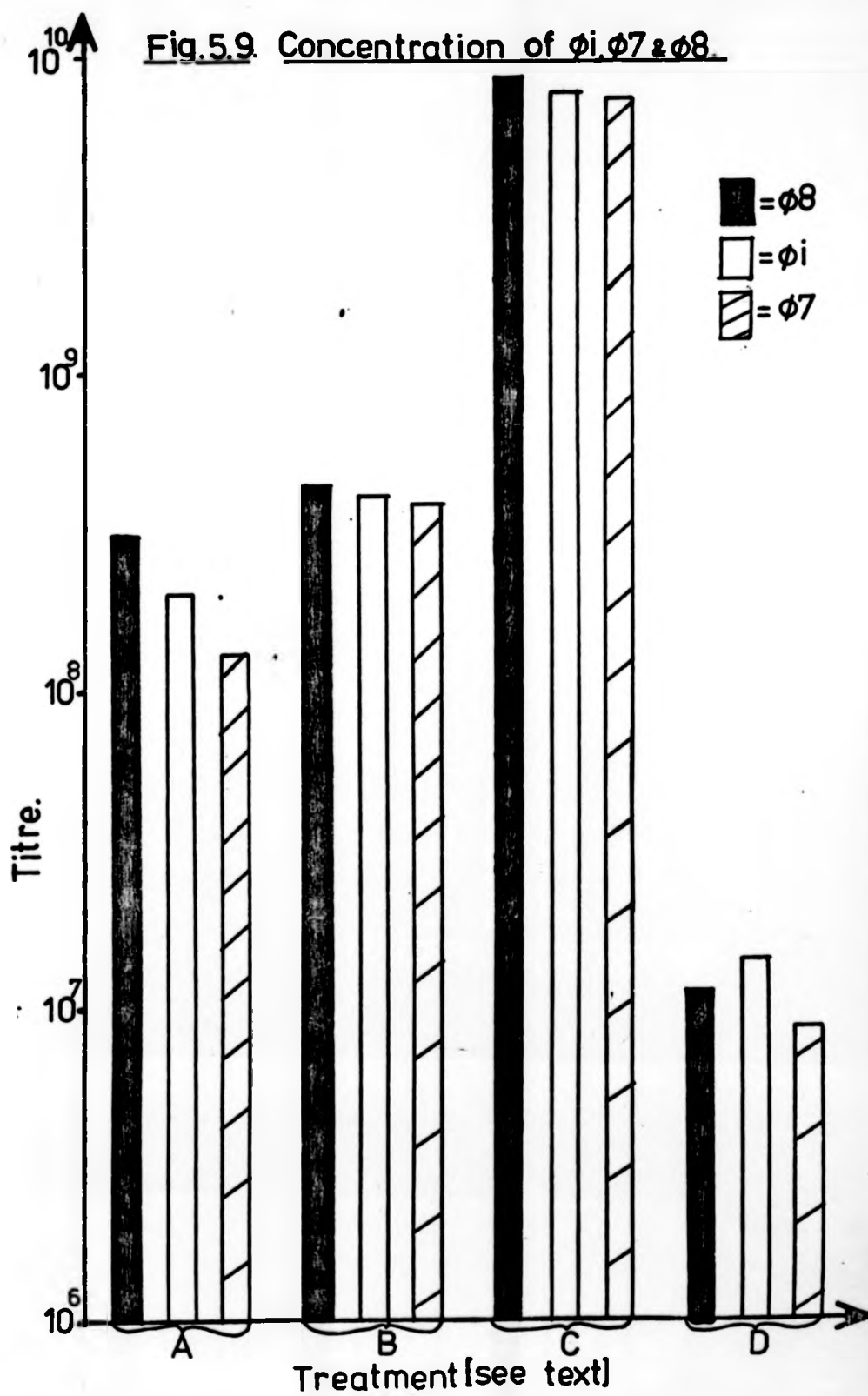
Table 5.21

Concentration of phages ϕ i, ϕ 7 and ϕ 8

PHAGE	TREATMENT	TITRE (p.f.u./ml).	%recovered relative to A.	%recovered relative to previous titre.
ϕ 8.Nu18	A.	3.0×10^8	100	-
	B.	4.4×10^8	147	147
	C.	9.5×10^9	3166	2159
	D.	1.1×10^7	3.7	0.1
ϕ i.Nu18	A.	2.1×10^8	100	-
	B.	4.1×10^8	195	195
	C.	7.9×10^9	3762	1927
	D.	1.4×10^7	6.7	0.2
ϕ 7.Nu18	A.	1.3×10^8	100	-
	B.	3.9×10^8	300	300
	C.	7.6×10^9	5846	1949
	D.	8.5×10^6	6.5	0.1

TREATMENTS

- A) Harvested after 30' contact between buffer and top agar.
 B) Harvested after overnight contact at 4°C .
 C) Post PEG/NaCl concentration.
 D) Post CsCl centrifugation and dialysis.



phase. At no time during these studies was a phage band visually detected after centrifugation. This is not surprising considering the low titres of phage lysates used here. However, using the same technology it proved possible to concentrate the virulent rhizobiophage, ØC.

These results mean that the phage was being lost or inactivated either during centrifugation or in dialysis, or both. When samples from the gradient were recovered and titred post-centrifugation the recovery varied from 10% to 89% of the input titre. The reason for the variation was unknown but similar results were obtained whether caesium chloride or metrizamide was used. This did suggest, however, that the treatment responsible for the "inactivation" of the infectious phage was the dialysis rather than the density gradient centrifugation.

The effect of dialysis on phage recovery

To ascertain whether or not dialysis against phage buffer was responsible for the reduction in titre of these phage lysates, the following simple experiment was done. Three lysates of Ø1, Ø7 and Ø8, respectively, were dialysed against phage buffer for 2 hours then titred. The results are presented in Table 5.22. The data show that the dialysis step is responsible for the drop in titre of the lysates. Also, it seems likely that the volume against which the lysate was dialysed affected the final recovery because the lysate dialysed against the largest volume, Ø8, had the lowest survival. There could be several explanations for this inactivation effect.

Perhaps there is some toxic compound in the visking dialysis tubing which inactivates the phage. Another possibility is that because this was done at room temperature the rate of inactivation was increased. Alternatively, dialysis led to the loss of some small molecule from the phage lysate and this small molecule is required for phage stability or plaque forming activity. In such a case the plaque forming activity may or may not be regained on the

Table 5.22Dialysis of phage ϕ i, ϕ 7 and ϕ 8 lysates

Phage	Initial titre	Lysate Volume	Dialysis Volume	Final titre	% Inactivated
ϕ i	1.9×10^7	10ml.	2x2,000ml.	2.0×10^6	90
ϕ 7	1.5×10^6	10ml.	1x1,000ml.	4.0×10^5	74
ϕ 8	1.0×10^7	10ml.	3x2,000ml.	1.4×10^5	99

addition of the small molecule back to the post-dialysis lysate. Finally, dialysis may lead to a concentration, in the lysate, of a small molecule from the buffer which can inactivate the phage. However, the latter explanation is unlikely because, when serial dilutions of the phage lysates were made into phage buffer, they retained their plaque forming activity.

Is there a toxic compound in the dialysis tubing and is phage inactivation temperature dependent?

To determine whether temperature or toxicity in the tubing, was an important factor in phage inactivation the following experiment was done. Four 10ml. lysates of Ø7 at 1.6×10^9 pfu/ml. were treated as follows:-

- 1) The lysate was kept at 4° but not in contact with dialysis tubing i.e. non dialysed control.
- 2) The lysate was held in visking tubing at room temperature but not in contact with phage buffer. i.e. no dialysis.
- 3) The lysate was held in visking tubing at room temperature and dialysed against 500 ml. of phage buffer.
- 4) The lysate was held in visking tubing at 4° and dialysed against 500 ml. of phage buffer.

All lysates were treated as above for 2 hrs. before titration. The results of the titrations are presented in Table 5.23.

The data show that there was no "toxicity" in the dialysis tubing because no inactivation of phage occurred on exposure of the lysate to the tubing. Neither does the temperature appear to have had any effect on the loss of plaque forming activity. However, whenever the lysates were dialysed against the phage buffer a dramatic reduction in plaque forming activity occurred and this was unaffected by temperature. These results mean that phage inactivation must either be due to the loss of some small molecule from the lysate or an increase in the concentration of some small

Table 5.23 Effect of temperature and dialysis tubing on phage
recovery

Treatment	Titre after treatment	titre relative to initial
1.	1.5×10^9	~ 1
2.	1.36×10^9	~ 1
3.	2.5×10^7	1.56×10^{-2}
4.	2.6×10^7	1.6×10^{-2}

for treatments see text

molecule after dialysis.

Dialysis against yeast extract solutions

If the loss of a small molecule from the lysate is the cause of phage inactivation then it may be possible to reactivate the phage by the addition of that molecule to the post-dialysis lysate. Finding such a molecule may be difficult however. By the same logic, if the said molecule is present in the dialysis buffer to the same concentration as it is in the lysate then no net loss of the molecule would occur from the lysate.

A Ø7 lysate was divided into five 5 ml. volumes and the samples were treated thus:-

- 1) The lysate was held in dialysis tubing, without dialysis, at 4° overnight.
- 2) The lysate was held in dialysis tubing at 4° overnight and dialysed against 450 ml. of 1% yeast extract at pH 6.9.
- 3) The lysate was treated as in 2) but dialysed against 0.1% yeast extract at pH 6.9.
- 4) The lysate was treated as in 2) but dialysed against 0.01% yeast extract at pH 6.9.
- 5) The lysate was treated as in 2) but dialysed against 0.001% yeast extract at pH 6.9.

All lysates were titred after overnight dialysis. The results are presented in Table 5.24. Even at high yeast extract concentration (1%) there was a reduction in the titre of Ø7 recovered. The phage loss did not increase with increased dilution of the yeast extract. It must be assumed then that if the decrease in titre is due to the loss of a small molecule from the lysate, that that molecule is not present to any significant degree in yeast extract. It has been known for many years that certain "co-factors" exist without which certain viruses show a reduced plaque forming activity

(Anderson, 1945; 1946). However the discovery of the particular requirement can be difficult. From the data in Table 5.24 it is interesting that about 90% of the phage was inactivated after each treatment. The dilution factor was of the order of 1:91.

Table 5.24 Effect of dialysis against yeast extract solutions on
phage recovery

Treatment	Titre after treatment	titre relative to initial
1.	1.0×10^7	1
2.	1.2×10^6	1.2×10^{-1}
3.	1.2×10^6	1.2×10^{-1}
4.	0.9×10^6	0.9×10^{-1}
5.	1.0×10^6	1.0×10^{-1}

Diaflo ultrafiltration

In an attempt to find a method of concentrating these phages without using dialysis a lysate of ϕ i.Nu18 was subjected to ultrafiltration. The data presented in Table 5.25 show the recoveries of phage from each part of the filtration.

The first point is that about 1% of the input phage managed to get into the effluent. This may have happened by passage of the phage through small holes in the filter or passage around the sides of the filter. Because the filter used excludes molecules of 300,000 daltons it is unlikely that the phage had passed through the pores of the filter. It is interesting that the recovery of phage from the effluent here was more than the final recovery of phage from the previous series of experiments on centrifugation and dialysis!

The total recovery in the residue was 56% of the input phage titre. However, this result appears better than it is in practice. Because of the pronounced slime content of the samples they were effectively useless for any experimental work. Nevertheless this suggested that ultrafiltration may be useful for the concentration of these phages if the lysates could be freed of their slime prior to, or after, ultrafiltration. Over the course of several experiments the recovery in the residue varied from 10% to 70% of the input titre, but the slime was always present. It was found that the phage could be purified from the slime by CsCl density gradient centrifugation but, again, dialysis of the lysate led to a reduction in titre.

Table 5.25Recovery of phage after ultrafiltration

SAMPLE	VOLUME	TITRE/ ml.	TOTAL PHAGE	% of INPUT
INPUT	75ml.	1.5×10^9	1.13×10^{11}	100
EFFLUENT	65ml.	2.1×10^7	1.37×10^9	1.2
SLIME LAYER	3ml.	1.5×10^{10}	4.5×10^{10}	39.8
SUPRA-SLIME LYSATE	7ml.	2.59×10^9	1.8×10^{10}	16.0

What is the source of $\phi 7$ and $\phi 8$?

How does $\phi 7$ and $\phi 8$ arise from the interaction of ϕi and Su298? It seems reasonable to assume that if genetically marked ϕi could be isolated that the appearance of the marker in the "progeny" phage could be followed. If, for example, a temperature sensitive (t.s.) mutant of ϕi was used in the induction of $\phi 7$ and $\phi 8$ the possible inheritance of the t.s. marker into $\phi 7$ and/or $\phi 8$ could yield information about the genetic source of these phages. To test such a hypothesis of genetic relatedness between ϕi and the other two phages, genetically marked strains were required. Attempts were made at isolating t.s. mutants of ϕi , $\phi 7$ and $\phi 8$ on Nul8, using UV light as the mutagen. Phage lysates were irradiated, and plated on Nul8 at 14°C . The survival of the phage ranged from 10^{-1} to 10^{-5} . All plaques which arose at 14°C were replicated onto seeded Nul8 lawns and incubated at 14°C and 30°C to screen for t.s. mutants. In the course of the study 450 ϕi plaques; 250 $\phi 7$ plaques and 250 $\phi 8$ plaques were replicated but no definite t.s. phenotypes were observed. All that was noticed was that $\phi 8$ plaques were smaller at 14°C than the plaques of ϕi and $\phi 7$. The reason for this is unknown.

Rather than continue this attempt at isolating t.s. phage mutants using another mutagen, use was made of the high frequency mutation, to the clear plaque phenotype, of ϕi . From earlier studies it was noted that clear plaques of ϕi arose on Nul8 at a frequency of 10^{-3} - 10^{-4} . Although the clear plaque/turbid plaque distinction on Nul8 was not as pronounced as on Su297 ($\phi 7$ - $\phi 7^{\text{C}}$) it was nevertheless reproducible. A clear plaque mutant of ϕi , ϕi^{C} . Nul8, was plaque purified on Nul8 and used in the induction of $\phi 7$ and $\phi 8$ from Su298. The resultant plaques were examined for the clear plaque phenotype. The induction was by the broth and plate

methods and the lysate produced was titred on the three hosts.

The results are presented in Table 5.26.

The data show that, although a clear plaque mutant of ϕ_i was used to induce ϕ_7 and ϕ_8 , all of the ϕ_7 plaques were turbid. Of course, clear plaques arose at a frequency of 10^{-3} - 10^{-4} but this is normal for ϕ_7 . Although only low numbers of ϕ_8 plaques were recovered by the broth induction they appeared to be clearer than the normal ϕ_8 phenotype. The ϕ_8 plaques produced by the broth method appeared to be more clear than those produced by the plate method which, in turn, were clearer than the normal ϕ_8 . However this assessment of clear and hyperclear was a very subjective one when compared with, say, the ϕ_7 - ϕ_7^C comparison on Su297.

Representative plaques of the hyperclear variant of ϕ_8 (ϕ_8^{hc}) were plaque purified on Su298 before titration on the other hosts. A clear plaque mutant of ϕ_8 formed on Su297 (ϕ_8^C .Su297) was also titred on the other hosts to compare its phenotype with that of ϕ_8^{hc} . The results are presented in Table 5.27.

The data show that the h.c. phenotype of ϕ_8^{hc} .Su298 was not expressed on Su297 although the clear plaques arose on Nul8. When the ϕ_8^C .Su297 lysate was titred on the other hosts it formed turbid plaques on Nul8 and turbid/clear plaques on Su298. Hence the clear plaque phenotype must be host dependent. The fact that ϕ_8^C .Su297 can only form turbid plaques on Nul8 is identical to the situation with ϕ_7^C .Su297 as found by Barnet (1968) and in this study. It must be borne in mind that ϕ_i is always present in Su297 lysates and this fact complicates the analysis of whether or not a phage grown on Su297 can or cannot grow on Nul8. It is interesting that ϕ_8^{hc} .Su298 could only plate on Su297 at a reduced frequency of 4.4×10^{-3} . The reason for this is unknown.

This host dependence of the clear plaque phenotype complicates

Table 5.26

Induction of Ø7 and Ø8 using Øi^C.Nul8

LYSATE	INDUCTION METHOD	TITRATION ON HOST		
		Su297	Su298	Nul8
Øi ^C .Nul8	BROTH	10 ⁷ T.P.	10 ¹ hcp.	N.T.
Øi ^C Nul8	BROTH	1.2x10 ⁵ T.P.	2x10 ⁰ hcp.	N.T.
Øi ^C .Nul8	PLATE	N.T.	1x10 ³ cp/TP	4x10 ⁸ c.p.

T.P. = turbid plaques

cp = clear plaques

hcp = hyper clear plaques *

cp/TP = intermediate between clear & turbid

N.T. = not tested.

* Ø8 plaques on Su298 are generally cp/TP but these plaques appeared to be more clear than usual. The assessment of such status is very subjective.

Table 5.27 Titration of $\phi 8^{hc}$ and $\phi 8^C$ on various hosts

Phage	TITRE ON HOST		
	Su298	Su297	Nu18
$\phi 8^{hc}$.Su298	1.7×10^9 hc	7.8×10^6 tp	1.0×10^9 cp
	e.o.p.=1	4.4×10^{-3}	0.6
$\phi 8^C$.Su297	2×10^1 cp/tp	1.3×10^8 cp	2.1×10^5 tp
	e.o.p.= $\frac{2}{7}$ 1.5×10^{-7}	1	1.6×10^{-3}

the analysis of the inheritance of the original clear marker of ϕ_i in the production of ϕ_7 and ϕ_8 . Therefore the clear plaque marker is of no great utility in determining the inheritance of ϕ_i genes by ϕ_7 and ϕ_8 . Although the ϕ_8 plaques produced by such inductions appeared to inherit the clear plaque marker their numbers were very low. Also, the difference between the clear, hyperclear and turbid status of plaques on Su298 was so subjective that these data cannot be considered unequivocal.

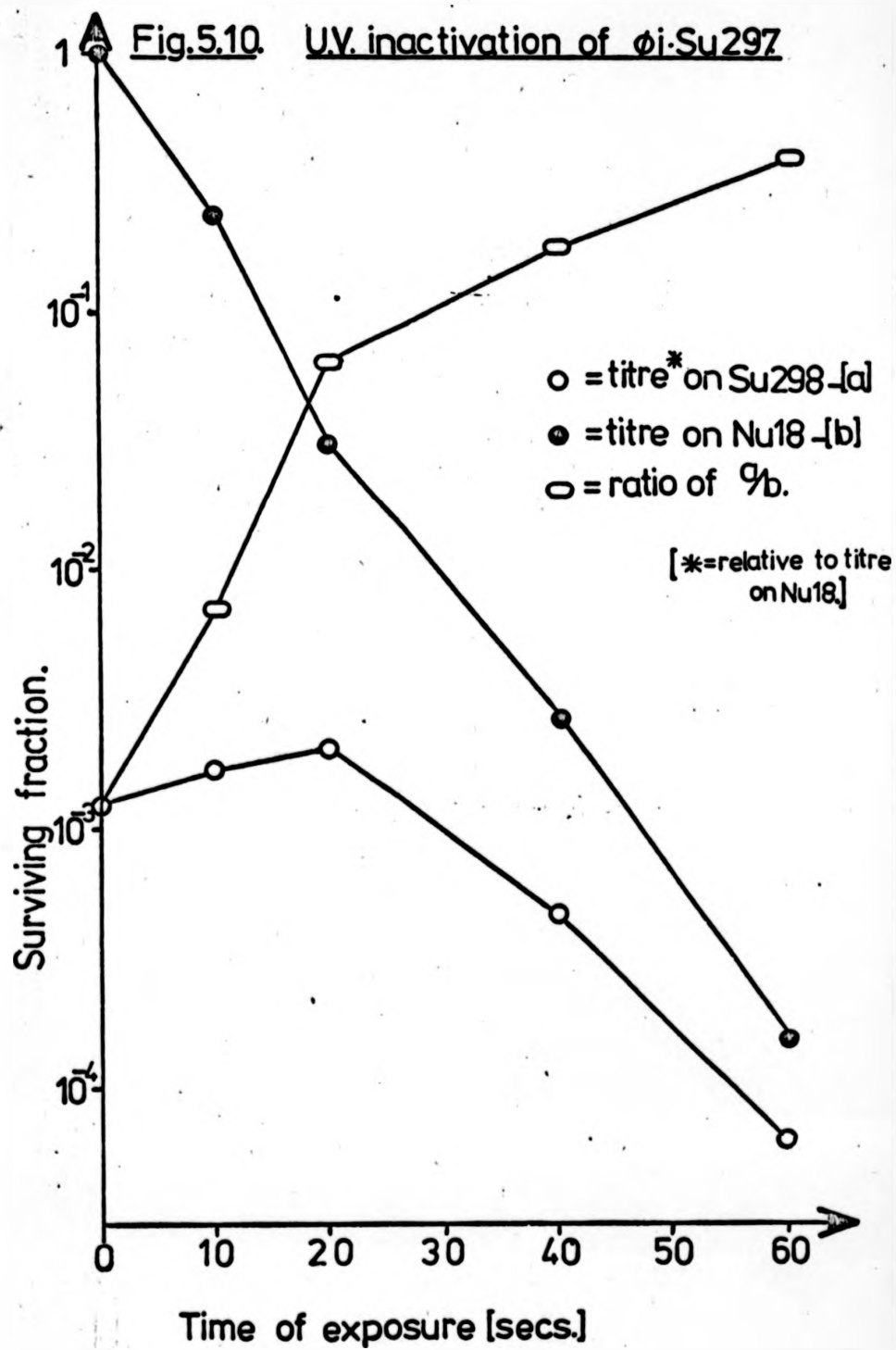
The fact that Su297 has at least one resident prophage which is probably related to ϕ_7 and ϕ_8 may affect the expression of the clear plaque phenotype of ϕ_8^{hc} .Su298 on Su297. A better marker may be to use amber phage mutants of ϕ_i in combination with amber suppressor and suppressor free hosts. In such a system it would be possible to block the development of an amber mutant of ϕ_i in a suppressor free host. The inheritance of the amber marker could be followed into ϕ_7 and ϕ_8 . Unfortunately, to date, no amber phage mutants or amber suppressor hosts of Rhizobium have been isolated. It may prove possible to isolate such amber suppressor hosts however, using the technique developed by Mindich et.al. (1976). The construction of amber phage mutants and amber suppressor hosts would be of great help in the analysis of this complex system.

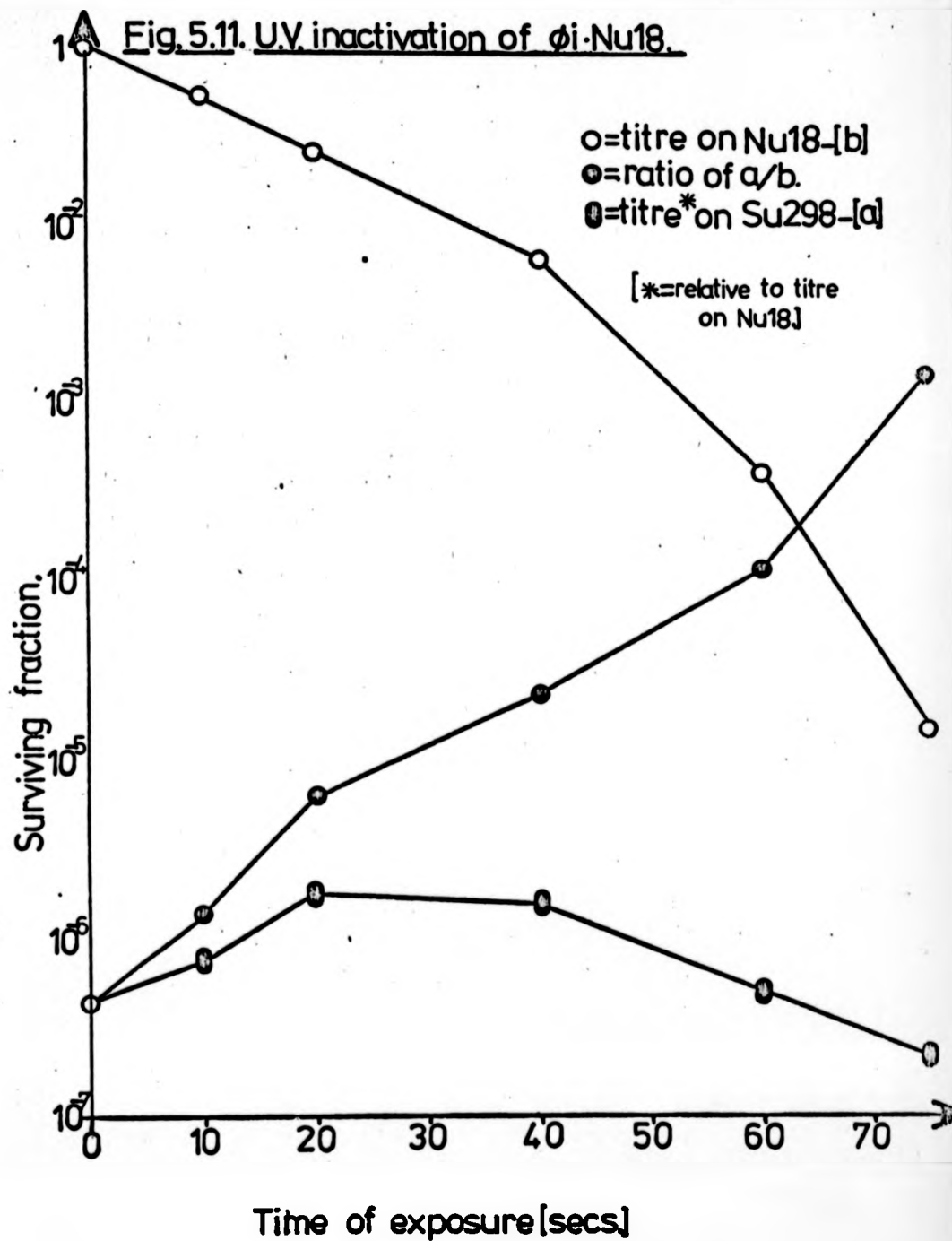
The effect of UV irradiation of ϕ i on the induction of ϕ 8

It has been known for many years that in-vitro irradiation of phage can increase the recombination between the irradiated phage nucleic acid and resident prophages in the infected cell (e.g. see Yamamoto, 1967; Hart and Ellison, 1970; Blanco and Devoret, 1973; Lin and Howard-Flanders, 1976). Based on this knowledge, lysates of ϕ i.Su297 and ϕ i.Nul8 were irradiated and titred at intervals on Su298 and Nul8 to quantify the titre of ϕ 8 and residual ϕ i. These experiments were performed several times and yielded the same results each time. Representative data are presented in Figures 5.10 and 5.11.

The plaque forming ability of ϕ i on Nul8 dropped by about 3 logs after 50 seconds irradiation. This has been described before (see ~~pages 167-169~~). The inactivation was approximately linear as plotted on a semi-log scale. However, the plaque number on Su298 increased slightly after low UV dosage and gradually declined thereafter. This effect, although slight, was reproducible. When the ratio of plaque number on Su298 to Nul8 is plotted against increasing dosage (time) it can be seen that the ratio increases progressively, up to 60 seconds. From these data it is possible to argue that there are two phages present in the ϕ i.Su297 lysate. One of these phages could be responsible for plaques on Nul8 and is more radio-sensitive than the other phage which plaques on Su298 i.e. ϕ i.Nul8. If such a situation was true then one would not expect to be able to repeat the experimental results using plaque purified ϕ i.Nul8. However, the data represented in Figure 5.11 show that a similar trend did occur when ϕ i.Nul8 was used. There were some differences, however, in the kinetics. The rate of ϕ i inactivation departs from linearity, at higher UV dosages. The reason for this is unknown but may be a reflection of debris in the lysate.

Fig.5.10. U.V. inactivation of ϕ i-Su297





Because of the apparent increase in the ϕ i.Nul8 inactivation rate at high UV dosage the ratio of plaque number on Su298 to Nul8 is increased at higher UV dosage. This may be an artefact of the system. However, the important feature; namely stimulation of ϕ 8 production at low UV dosage, is present in both experiments.

As recorded before, ϕ i.Nul8 forms plaques on Su298 at an e.o.p. of 10^{-5} whereas ϕ i.Su298 does so with an e.o.p. of 10^{-3} relative to the titre on Nul8. Hence, whatever the effect the UV light is having on the system it is manifesting its effect via ϕ i. As a control, an Nul8 culture was lysed and irradiated according to the protocol used for ϕ i.Nul8. As expected this irradiated, phage free, control had no effect at all on ϕ 8 formation.

There are several possible interpretations of these data, in the absence of further experiments. It is well known that UV light is mutagenic to phage and bacteria. It is therefore possible to argue that, as in the Barnet model, the plaques on Su298 are due to spontaneous virulent mutants of ϕ i. Hence the mutagenic effects of UV on ϕ i would stimulate the formation of ϕ i virulent mutants. However, as shown earlier, these plaques on Su298 are not ϕ i^{vir} mutants. Also, if this stimulation of plaque formation on Su298 were due to the increase in mutant phage formation one might expect there to be a corresponding increase in the proportion of clear plaque mutants of ϕ i on Nul8 with increasing UV dosage. Finally, it would be expected that the formation of plaques on Su297 would occur at higher UV dosage as ϕ i^{vir} mutants may be formed. Neither of these events occurred in the course of these experiments. It seems reasonable to conclude therefore, that the stimulation of plaque formation on Su298 by in-vitro irradiation of ϕ i is not due to any mutagenic effects of UV but must be due to some other effect.

It is known that the introduction of in-vitro irradiated DNA

into a host cell can have a variety of effects. Such effects include the stimulation of recombination (e.g. see Cordone et.al., 1975; Lin and Howard-Flanders, 1976), the inhibition of cell division (Devoret and George, 1967; Monk, 1969), and the induction of resident prophages (Rosner et.al., 1968).

In rhizobial research UV irradiation has been used to enhance recombination between transducing phage DNA and the resident bacterial genome in R.meliloti (Svab. et.al., 1978) and R. leguminosarum (Buchanan-Wollaston, 1979). It seems likely in this system that either the UV is inducing recombination or it leads to the induction of resident prophages in Su298. Because mitomycin or UV do not cause the development of $\phi 7$ or $\phi 8$ in Su298 without the action of ϕi it seems unlikely that resident prophages are being induced from Su298. Hence, although the evidence is purely circumstantial, it is plausible that UV damaged ϕi can stimulate the formation of $\phi 8$ due to its effects on recombination. However, more detailed experiments are required to test this hypothesis.

Discussion of the various models for the biogenesis of $\phi 7$ and $\phi 8$

Takahashi and Quadling (1961) suggested that the phage responsible for the production of $\phi 7$ and $\phi 8$ was defective because it could not form plaques on any host which they tested. Barnet (1968) showed that ϕi was a plaque forming phage, at least on Nul8, and so was not defective. Both Takahashi and Quadling (1961) and Barnet (1968) suggested that Su298 is lysogenic for a defective prophage and both publications favoured the hypothesis that $\phi 7$ and $\phi 8$ arose as a result of recombination between ϕi and the presumed defective prophage in Su298. The results obtained by Barnet, and in this study, show that the two obligate requirements for $\phi 7$ and $\phi 8$ production are ϕi and viable Su298 cells. It is difficult to envisage how two novel, heteroimmune, phages can be produced from this phage/host interaction without invoking the presence of at least one prophage, viable or defective, in Su298. The fact that ϕi , $\phi 7$ and $\phi 8$ cannot be definitely differentiated by UV inactivation kinetics or thermal inactivation kinetics suggests that they are closely related and, indeed, from the studies of Barnet and from this study, the only reproducible differentiating feature of these phages is their differences in host range. This study has accentuated the need for a molecular biological analysis of these phages by the methods of DNA-DNA heteroduplex analysis and coat protein analysis. However, although the titres of lysates used in this study were, on average, about 2-3 logs higher than those used by Barnet, it proved impossible to generate lysates of high enough titre for a more molecular analysis of these phages. Because of this problem, the only method left by which to examine the relationships between the three phages was by using genetically marked ϕi as the inducing agent for $\phi 7$ and $\phi 8$. When ϕi^C was used to induce $\phi 7$ and $\phi 8$ the $\phi 7$ produced did not inherit the clear phenotype of the "parental"

ϕ_i^C although the ϕ_8 plaques produced on Su298 were more clear than the plaques normally formed by ϕ_8 . Such ϕ_8^{hc} .Su298, though, yielded turbid plaques on the bilysogenic Su297 and so it could not be said with certainty that the ϕ_i^C marker had been inherited by ϕ_8 . Further, the bilysogenic status of Su297 complicates the overall analysis of this result. Consequently, there is no substantial evidence for a recombinational event leading to formation of ϕ_7 and ϕ_8 .

Nevertheless, if a recombinational event between ϕ_i and a genetic element in Su298 were responsible for the generation of ϕ_7 and ϕ_8 , then it would be expected that low dosage irradiation of ϕ_i or Su298 prior to infection would lead to a stimulation of ϕ_7 and/or ϕ_8 synthesis. When ϕ_i was subjected to in vitro irradiation, prior to titration on Su298, the number of ϕ_8 plaques formed actually increased after low UV dosage. As would be predicted from the results of other studies (e.g. see Lin and Howard-Flanders, 1976; Buchanan-Wollaston, 1979) the number of ϕ_8 (recombinant) plaques formed reached a peak then gradually declined. It was not known if the UV irradiation stimulated the synthesis of ϕ_7 to the same extent as ϕ_8 . It is, however, difficult to reconcile these data with any of the epigenetic or physiological models for ϕ_8 biogenesis. This evidence, therefore, may suggest that the synthesis of ϕ_8 is stimulated by recombination between ϕ_i and some genetic element(s) in Su298. Only further experiments can prove or disprove this recombinational model. One prediction of this model could be that low dosage UV irradiation of Su298, prior to ϕ_i infection, would lead to a stimulation in the synthesis of ϕ_8 .

According to Barnet's model ϕ_8 was a virulent mutant of ϕ_i and ϕ_7 was a recombinant between ϕ_i and a heteroimmune defective prophage in Su298. It has been shown in this study that ϕ_8 cannot be a virulent mutant of ϕ_i . In Barnet's model, ϕ_7 acquired the

immunity of the defective prophage from Su298 and thus could plate on Su297 but not Su298. This model explained the data obtained by Barnett because she could neither isolate Su297(8) lysogens nor find plaques on Su298 from $\phi 7$.Su297. However, this study has shown that $\phi 8$ is a temperate phage in Su297 and that $\phi 7$.Su297 lysates will form plaques on Su298, albeit at low frequency. Whether or not these latter plaques are due to $\phi 7$ or ϕi cannot be said with certainty but the fact that $\phi 8$ can lysogenise Su297 means that $\phi 8$ cannot be ϕi ^{vir}. Consequently, a model has to be developed in which $\phi 8$ and $\phi 7$ can lysogenise Su297. In this context it is remarkable that there are many similarities between the phenomenon of the generation of two novel phages in this system and the data obtained from studies on recombination between two Salmonella phages, P22 and L (Bezdek and Amati, 1968).

The bipartite immunity control model

This model was originally proposed by Bezdek and Amati (1968) and it was based on the following. Two phages can be genetically closely related but may differ in their immunity specificity. If these phages are subject to more than one, genetically distinct, heteroimmune repression system, then crosses between them would be expected to yield temperate recombinants which have altered immunity properties. However, where only one immunity locus is involved, recombination between two heteroimmune but genetically related phages cannot yield two phages with completely novel immunity properties.

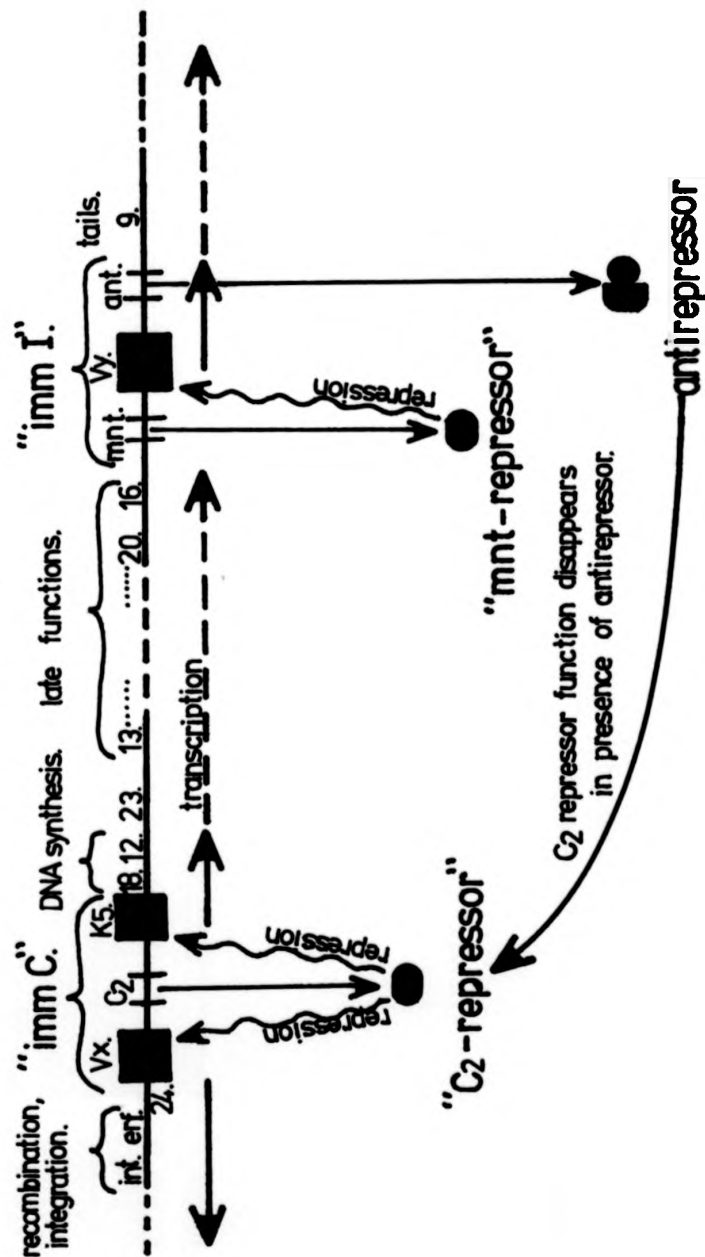
The task of Bezdek and Amati, and correspondingly of this study, was to develop a model to explain the biogenesis of two novel, heteroimmune, temperate phages from a recombinational event between two related but heteroimmune phage parents. The model invoked the possession, by the original genetically related but heteroimmune phages, of two immunity regulator systems. The two phages studied

were P22 and L and both plated on Salmonella typhimurium. Both phages are related by partial genetic homology (Bezdek and Amati, 1967) but are heteroimmune and can recombine. Among the progeny phages there is always a class of temperate recombinants which are heteroimmune with either of the parentals. Two plaque types, one more frequent than the other, were generated on one particular strain of Salmonella typhimurium. It was found that the minor class of phage plated on a host, lysogenic for either of the parental phages, with equal efficiency. However, the other class of phage could only plaque on one lysogen and failed to grow on the other lysogen (e.o.p. $<10^{-6}$).

The two immunity regions of these related phages were designated $I_{22}C_{22}$ and I_L and C_L for P22 and L respectively. After recombination two novel heteroimmune phages were generated of the immunity classes $I_{22}C_L$ and I_LC_{22} . In an attempt to explain these findings Bezdek and Amati reasoned that the product of one of the immunity genes could act on the receptor site and repress the other immunity gene in the same phage. i.e. the I_{22} product was active on the I_L site.

Subsequently, the model of Bezdek and Amati (1968) was refined and developed into a model of bipartite control of repression and immunity (Botstein et.al., 1975; Levine et.al., 1975). According to this later model the imm C gene cluster is responsible for the repression of transcription of "lytic cycle" function genes and it acts in an analogous fashion to the λ repressor (Botstein et.al. 1975). Unlike λ control, however, P22 is subject to the superimposition of a further level of control of repression from the product of the imm I gene cluster. The product of this imm I cluster is called the anti repressor which acts on the C_2 repressor of imm C in such a way as to circumvent repression and thereby allow lytic gene derepression (see Figure 5.12). Hence the imm I gene cluster plays a dominant role in the ultimate control of gene expression in the phage.

Fig.5.12. Bipartite immunity control model [P22]. (Botstein et. al. 1975.)



A model very similar to this can be invoked to explain the biogenesis of $\phi 7$ and $\phi 8$ from ϕi and a defective prophage in Su298. It is known that Su297 and Su298 are related (Vincent, 1962) and from such a background it is not unreasonable to think that the presumed defective prophage (ϕx^d) in Su298 could be genetically related to ϕi . If ϕi and ϕx^d are both subject to bipartite immunity control as in P22 and L then recombination between these two could generate two novel heteroimmune phages. By analogy with the P22/L system found by Bezdek and Amati, one of these recombinant phages would be expected to plate on lysogens of either parental phage whereas the other recombinant phage would only be able to plate on a lysogen of one of the parental phages. In the Bezdek and Amati model the strain made lysogenic for either parental phage was the same (LT2). Whereas in the Su297/Su298 system two related but distinct strains are the source of the parental phages. This being the case, there are other factors such as host controlled restriction which may exert an additional effect on the e.o.p. of the parental and recombinant phages on the Su297 and Su298 strains.

As represented in Fig. 5.13, ϕi and ϕx may be regarded as subject to dual immunity control. On induction ϕi will be produced as the repressors are inactivated and plaque forming ϕi will be released from Su297. However, it must be assumed the ϕx in Su298 is defective since no phage particles have ever been detected after UV or MC induction of Su298. The defect in ϕx^d must be assumed to be either a deletion, double mutation or nonsense mutation in an essential gene(s). Recombination between these phages could lead to the formation of a variety of phages including the classes depicted in Fig. 5.14.

A recombinational event occurring between the immunity regions could lead to the formation of type a) phage as well as the reciprocal

Fig. 5.13 ϕi and ϕx^d as dual immunity lysogens.

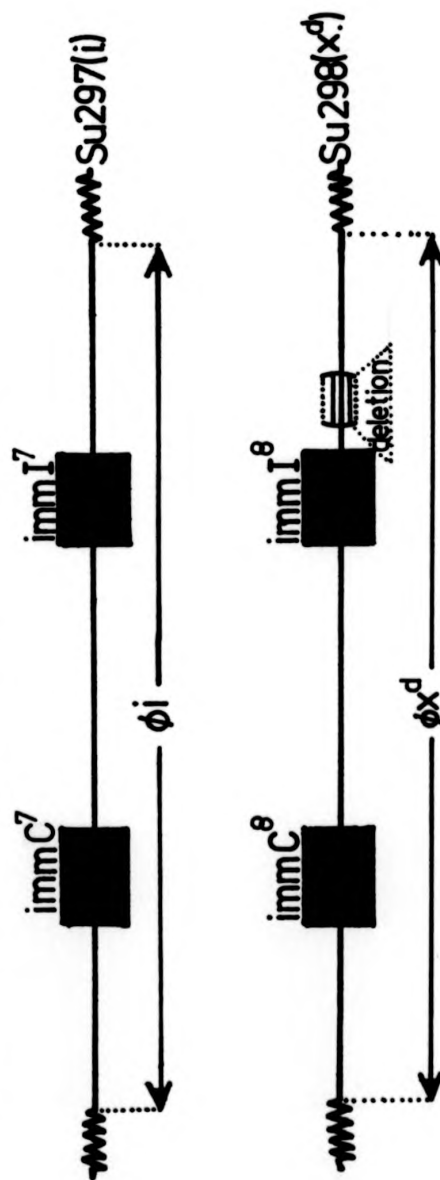






Fig. 5.14 Progeny classes of $\phi_i \times \phi_x^d$

		no. of cross- overs.	phage.
a)		1	ϕ_7 .
b)		1.	defective.
c)		2.	ϕ_8 .
d)		1.	ϕ_x .

type b. If a deletion exists in the ϕx^d phage, then, as shown in Fig. 5.14, this defect would manifest itself in the class b phage. However, if in addition to the first type of recombinational event, a second crossover were to occur at a site between the imm I site and the deletion of ϕx^d it may be possible to generate a plaque forming phage of the immunity class c. Finally, if only the latter crossover event was to occur then a functional phage of class d could be formed.

By analogy with the bipartite immunity control model as expressed for P22 and L the following statements could hold true:-

1. Lysogens are immune to a superinfecting phage if
 - a) prophage and superinfecting phage have both imm C and imm I the same, or
 - b) the imm I regions are different but the prophage carries the imm I gene of ϕx .
2. Lysogens are sensitive to superinfecting phage if
 - a) the imm C regions are different, or
 - b) the imm C regions are the same and the imm I regions are different but the prophage carries the imm I of ϕi .

Based on these premises then the recombinant classes from the ϕi and ϕx^d mating would be expected to have the host ranges as shown in Table 5.28. In summary, the model leads to the following predictions.

1. ϕi would be expected to plaque on Su298 but not Su297.
2. Because of the defect in essential gene(s) in ϕx^d neither ϕx^d nor any derivatives carrying the defect could form plaques on any of the hosts.
3. The phage designated $\phi 7$ would have the immunity genes imm C imm I and so would be expected to plate on Su297. However this phage could not plate on Su298 because of the homoimmune

Table 5.28 Predicted host range of ϕ_i/ϕ_x^d recombinant classes

PHAGE	IMMUNITY	Su297	Su298	Su297(7)	Su297(8)
		(<u>immC₇</u> <u>immI₇</u>)	(<u>immC₈</u> <u>immI₈</u>)	(<u>immC₇</u> <u>immI₇</u> <u>immC₈</u>)	(<u>immC₇</u> <u>immI₇</u> <u>immI₈</u>)
ϕ_i	<u>immC₇</u> <u>immI₇</u>	-	✓	-	-
ϕ_x^d	<u>immC₈</u> <u>immI₈</u> ^d	-	-	-	-
ϕ_7	<u>immC₈</u> <u>immI₇</u>	✓	-	-	✓
ϕ_8	<u>immC₇</u> <u>immI₈</u>	✓	✓	✓	-
ϕ_x	<u>immC₈</u> <u>immI₈</u>	✓	-	✓	✓

- = phage will not plate on this host

✓ = phage should plate on this host

imm C regions and the repression of the incoming phage imm I region by the imm I gene product of the resident ϕx^d .

4. The phage designated $\phi 8$ would be expected to plate on both Su297 and Su298. The phage can plate on Su298 because $\phi 8$ and the ϕx^d prophage have distinct imm C regions. But $\phi 8$ could also plate on Su297 because, although the imm C regions of $\phi 8$ and ϕi are identical, the imm I regions are different and the prophage ϕi carries the imm I gene of ϕi which cannot repress the expression of the imm I antirepressor of $\phi 8$.

Although $\phi 8$ is expected to plate on Su297 and Su298, from the model, the generation of $\phi 8$ is dependent on a double crossover event occurring between ϕi and ϕx^d . Hence one would expect that the frequency of formation of $\phi 8$ would be less than that of $\phi 7$ formation. This is the observed result.

5. The phage designated ϕx would be expected to plate on Su297 because the superinfecting ϕx and the ϕi prophage have heteroimmune imm C regions. However, Su298 would be ϕx resistant because of the dual homimmune repression exerted on ϕx by the resident defective ϕx^d .

The definition of $\phi 7$ in the studies of Takahashi and Quadling (1961), Barnet (1968) and in this study, have been purely on the grounds that it formed plaques on Su297 at a higher frequency than those on Su298, directly from specific induction of Su298 by ϕi . This model for the formation of $\phi 7$ and $\phi 8$ therefore predicts that there are two theoretical classes of " $\phi 7$ " as defined by this criterion of host range.

6. Based on the premises of the model it would be predicted that $\phi 7$ could plate on Su297 (8) but not on Su297 (7).
7. Conversely $\phi 8$ should be able to plate on Su297 (7) but not Su297 (8).

8. Finally, ϕ_x should be able to plate on both Su297 (7) and Su297 (8).

How then, do the predictions of the model agree with the observed results? The bipartite immunity control model predicts that ϕ_i should form plaques on Su298. Although ϕ_i can form plaques on Su298 it does so with a low e.o.p. (10^{-3} of ϕ_i assay on Nul8). Furthermore, these plaques on Su298 are not due to the lytic growth of ϕ_i but the formation and growth of a novel phage, ϕ_8 . However, if the growth of ϕ_i on Su298 was prevented due to a restriction system in Su298 then plaques of ϕ_i would not appear on Su298. That Su298 restricts ϕ_i was previously suggested by Barnet (1968).

From the model, only ϕ_8 can form plaques on both Su297 and Su298. Although Barnet found a reciprocal 3 log restriction of ϕ_8 between Su297 and Su298 this result could not be repeated in this study. However, the results of this study show that ϕ_8 .Su298 plates as efficiently on Su297 as on Su298. Hence Su297 does not restrict ϕ_8 . However, when ϕ_8 was grown on Su297 it could not grow back on Su298, yet the model predicts that ϕ_8 .Su297 should grow on Su298. But if a restriction system operates in Su298 then Su297-modified ϕ_8 would be subject to that restriction. Correspondingly, the phage designated ϕ_x would not be expected to grow in Su298 as, on superinfection, it would be subject to both restriction and homo-immune repression. Hence the model is in reasonable agreement with the data if one assumes that Su298 is a restricting host.

The model also predicts that ϕ_7 and ϕ_8 should be able to plate on Su297 (8) and Su297 (7) respectively but the experimental results show that Su297 (7) (Barnet, 1968; this study) and Su297 (8) (this study) are resistant to ϕ_7 , ϕ_8 and ϕ_7^C . However, it has been shown that Su297 synthesises a new somatic antigen when it is lysogenised by

Ø7 and it has been speculated that this synthesis correlates with the failure of Ø7, Ø8 and Ø7^C to adsorb to the Su297 (7) cell surface, (Barnet, 1968; Barnet and Vincent, 1969). The phage conversion phenomenon in Su297 only occurs when Ø7 lysogenises Su297 and so Øi itself cannot be responsible for the effect. There is circumstantial evidence from this study that Ø8 may have a similar effect to Ø7. Perhaps, as with P22, these phage may express a surface exclusion gene but only in the background of a Øi lysogen.

The prediction of a bipartite immunity control model for Øi and the hypothetical defective prophage can be used to explain many of the observations of the biogenesis and host range of Ø7 and Ø8. Nevertheless other phenomena have to be involved to explain other aspects of the data; namely restriction by Su298, and phage conversion by Ø7 and Ø8. Clearly, at this stage of the analysis of this intriguing system there are too many assumptions which have to be made to explain the biogenesis of Ø7 and Ø8. Hence the value of the applicability of the bipartite immunity control and recombinational models to this system will only be revealed by further experiments.

CHAPTER VI

TRANSFECTION AND TRANSDUCTION

IN R.trifolii

"Nothing but new whys and wherewithals"

Friedrich Nietzsche.

"What's beyond logic happens beneath will"

e.e. cummings.

"In order to arrive at what you do not know
You must go by a way which is the way of ignorance"

T.S. Eliot.

"It is essential to know the chill of all the objections
That come creeping into the mind, the battle between opposing ideas
Which gives the victory to the strongest and most universal
over all others"

Hugh MacDiarmid.

As discussed in the general introduction, the standard of rhizobial genetics, prior to the publication by Beringer and Hopwood, (1976), left a great deal to be desired. The validity of any genetical experiment rests heavily on the standard controls included within it and it was an unfortunate, but prominent, feature of most of rhizobial genetics prior to 1976, that controls were either inadequate or completely non-existent ! Why the rhizobial geneticists should have worked in a scientific vacuum when the genetics of E.coli; Bacillus and Pseudomonas was relatively well advanced is both a total mystery and gross error. Consequently the absence of necessary controls in the pre-1976 papers is, in itself, enough to invalidate them scientifically.

Because of the developments in recombination mapping (Beringer and Hopwood, 1976; Meade and Signer, 1977; and Kondorosi et. al., 1977); the construction of R-primes (Johnston et. al., 1978) and, very recently, the construction of strains capable of site specific, directional, chromosomal mobilisation (Julliot and Boistard, 1979) the genetics of Rhizobium is now open for advanced exploitation. However until very recently a major problem continued to exist and that was that recombination analysis was not sensitive enough to resolve closely linked genes e.g., rif^R and str^R markers are very closely linked in R.leguminosarum and cannot be adequately resolved by recombination analysis (Johnston and Beringer, 1977). Genes which are so closely linked can be resolved by transformation or transduction (Hayes, 1968). Indeed, since the completion of this study, a method of both restricted (Svab et. al., 1978) and, very recently, generalised transduction (Buchanan-Wollaston, 1979; Casadesus and Olivares, 1979) has been developed in Rhizobium. The power of transductional techniques has been demonstrated elegantly, in the rhizobia, by Johnston et.al., (1978). These workers found that, using a transducing phage, they could co-transduce the ability to nodulate and an antibiotic resistance marker and, from their data, concluded that nodulation ability is plasmid mediated in Rhizobium. Hence, using phage mediated transduction,

these workers were able to provide strong evidence in favour of an earlier hypothesis (Higashi, 1967) that the specificity of rhizobial/legume interaction is indeed plasmid coded.

At the start of this study the only unequivocal demonstration of transduction in Rhizobium came from the work of Kowalski (1967; 1970 and 1974). Kowalski (1967; 1970) demonstrated that a number of R.meliloti phages could effect generalised transduction and that one phage (ϕ L5) could co-transduce leu with symbiotic effectiveness (Kowalski, 1974). Until this study was completed there was no demonstration of transduction in a species of Rhizobium, other than R.meliloti. Also, when generalised transduction was finally demonstrated in other rhizobial species (Buchanan-Wollaston, 1979) a curious effect was noted. Buchanan-Wollaston (1979) used two UV- irradiated, virulent phages (ϕ RL38 and ϕ RL39) to show co-transduction of the rif, str and spc alleles in R.leguminosarum, finally proving the suggestions from recombinational mapping that such genes were very closely linked in Rhizobium (Johnston and Beringer, 1979). Using these phages it was also shown that several markers could be transduced, including the cotransduction of auxotrophies and antibiotic resistance markers as well as P1 group R plasmids. However, although interspecific transduction from R.leguminosarum to R.trifolii occurred, no transduction from R.trifolii to R.leguminosarum was detected (Buchanan-Wollaston, 1979). The reason for this was unknown but it was unlikely to be due to restriction.

Before the discovery of transduction in R.leguminosarum and R.trifolii attempts were made in this study, to effect generalised transduction in R.trifolii using both virulent (ϕ C^{T616}) and temperate (ϕ 7, ϕ 7^c and ϕ 8) phages used in earlier parts of this study.

Transduction using virulent phages

It has been shown that the virulent coliphage T1 (Drexler, 1970) and the virulent mutant of P1 (see Miller, 1972) can be used as vectors of bacterial DNA in transduction. Also the virulent phage, ϕ Cr30, of

Caulobacter crescentus has been used as a generalised transducer (Ely and Johnson, 1977). Because of the lytic effects of a virulent phage, conditions have to be provided in transduction, which minimise the killing effects of the phage thereby allowing the growth of transductants. This can be achieved by UV inactivation of plaque forming activity (Ely and Johnson, 1977; Buchanan-Wollaston, 1979); the use of amber suppressible phage mutants (Drexler, 1970; Yasbin and Young, 1974); or the use of temperature-sensitive, missense mutants of the phage (Campos *et. al.*, 1978). Finally, in a situation where a virulent phage has an obligate requirement for a metal ion, for adsorption, then that metal ion can be removed from the medium, post adsorption, so that reinfection in transductants is reduced. P1 adsorption is Ca^{++} dependent and so after adsorption of the P1 to the cells, all Ca^{++} can be sequestered from the medium by the addition of citrate. Hence reinfection, and killing, of the transductants cannot occur in the absence of Ca^{++} .

In the case of T1, conditional lethal amber mutants of the phage can be used and plated on a recipient under restrictive conditions i.e., in a sup⁰ host. Drexler (1970) has developed this system using double amber mutants of T1 with suppressor plus and suppressor free strains as donor and recipient respectively. Although T1, like P1, is a generalised transducer it does transduce bio at a higher frequency than other chromosomal markers (Drexler and Kylberg, 1975; Drexler, 1977). P1 displays a transductional preference for certain genes too (Masters and Broda, 1971) but the reason for this is different from that preference shown by T1 for bio.

Because conditional lethal mutants of ϕC had been isolated (Atkins, 1973) this system of virulent phage mediated transduction was attempted in R. trifolii. Using auxotrophs, antibiotic resistant mutants and R^+ derivatives of W19, and a conditional lethal mutant of ϕC ($\phi\text{C}^{\text{T616}}$), attempts were made at transduction by making a lysate at 15°C and selecting for transductants at the restrictive temperature (30°C), on the appropriate plates.

Transduction using temperate phages

Several examples of temperate phage mediated transduction exist for a variety of bacterial genera e.g., $\phi 80$, λ and Plkc in E.coli; Pl in Shigella and Klebsiella, P22 in Salmonella (See Hayes, 1968) and $\phi 16-3$ in R.meliloti (Kowalski, 1967; Svab et. al., 1978). Transduction can be classified into two types which are differentiated by the amount of bacterial DNA which the transducing particles carry and the specificity of the genetic markers transduced. In generalised transduction (e.g., Plkc in E.coli) the major type of transductant arises because of infection by a phage particle carrying only bacterial DNA. Consequently such transductants do not give rise to H.f.t. lysates after treatment by inducing agents, because they have arisen by recombination between the incoming bacterial DNA and the homologous region of the resident chromosome. Hence such transductants are generally not lysogenic. In restricted transduction (e.g., λ in E.coli) the transductants generally arise due to lysogenisation by a plaque forming, or defective, phage vector for the particular gene studied. The vector usually arises by aberrant excision of the prophage from the chromosome thereby leading to the formation of phage particles which have "picked up" genes, at low frequency, adjacent to the phage attachment site. Transductants from such are therefore generally lysogenic and, on induction, lead to the formation of H.f.t. lysates.

In an attempt to define restricted transduction in R.meliloti. Svab et. al., (1978) have studied the temperate rhizobiophage $\phi 16-3$. This phage has a chromosomal integration site close to the cys-46 marker and cys⁺ transductants of $\phi 16-3$ can produce H.f.t. lysates which transduce cys at a frequency of 10^{-2} per p.f.u.. Also, $\phi 16-3$ can transduce cys⁺ by the formation of defective transducing particles. Transductants from such particles, if raised from multiple infection can give rise to H.f.t. lysates but singly infected transductants cannot give rise to H.f.t. lysates even after superinfection by mature phage (Svab et. al., 1978). It appears likely that the DNA from the latter defective particles can integrate

Transduction using temperate phages

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into the chromosome but cannot be excised. Phage 16-3 has a defined genetic map (Orosz and Sik, 1970; Sik and Orosz, 1970; Orosz et. al., 1973) and because the localisation of the prophage is known from co-transduction and recombinational mapping (Svab et. al., 1978) this system may now be open to development along the lines of the elegant genetic system developed for λ in E.coli (e.g., see Shimada et. al., 1972; Shimada et. al., 1973; Schrenk and Weisberg, 1975). It may also be possible to develop ϕ 16-3 as a rhizobial - gene cloning vector analagous to the λ derivatives (Murray et. al., 1974; Murray and Murray, 1975; Borck et. al., 1976; Murray et. al., 1977).

Although nothing is known of the genetics or molecular biology of the ϕ 7-related phages in SU297 they are known to be temperate in SU297 (Barnet, 1968; this study). Because, at the start of this study there was no published work on transduction in R.trifolii either by virulent or temperate phages, attempts were made to use the ϕ 7-related phages as transducing vectors in this species. The benefit of using temperate phages rather than virulent phages lies in the fact that less stringent conditions need to be provided in the transduction since less phage-induced killing occurs with the temperate phages.

Transfection/transformation in the rhizobia

As mentioned in the general introduction there have been several reports of transfection and transformation in the rhizobia; although the standard of such reports is highly controversial. Using DNA from the temperate phage ϕ 16-3 transfection was reported to occur in R.meliloti; in one case using spheroplasts (Staniewski et. al., 1971) and in another case using a helper phage (Kondorosi et. al., 1974). However, the highest efficiency of transfection obtained was 10^{-8} and there was no report of the induction of competence in the cells used.

To date it is generally believed that the conditions for transfection competence are the same as those for transformation competence (Notani and

Setlow, 1974; Benzinger, 1978). There are many benefits from having a transformation/transfection system in any organism including its use in fine structure mapping (Hayes, 1968; Benzinger, 1978). However, in recent times the use of transfection and transformation has taken on a greater importance as a method of constructing plasmid containing strains and recombinant phages for the purposes of genetic engineering (e.g., Borck et. al., 1976; Murray et. al., 1977; Benzinger, 1978). Ultimately, of course, it is hoped that genetic engineering will be feasible with the rhizobia and so the development of this capability will be of considerable importance. Unfortunately there are a great variety of conditions which affect the ability of any organism to be transfected, including nucleic acid concentration; the genetic constitution of the recipient cells; temperature, and the presence or absence of a variety of metal ions, enzymes and inhibitors (e.g., see Benzinger, 1978). Consequently it can prove extremely difficult to determine the exact conditions required by any particular strain, for transfection.

Because of this importance of transfection, attempts were made at developing transfection in R.trifolii. Both ϕC and $\phi 7^C$ were used since they produced large clear plaques on their respective hosts, W19 and SU297.

Materials and Methods

Strain Construction

For genetic experiments genetically marked strains were required and the following methods were used.

Isolation of auxotrophs

From the survival curve (see chapter II, Fig. 2.1), 5 minutes exposure gave about 30% survival and auxotrophs arose at a frequency of 1% of the survivors. Because Su297 does not produce much extracellular slime, replica plating proved to be very easy with this strain. Three mutants of Su297, produced by this method, were purified and their auxotrophic requirements determined. Each mutant reverted to prototrophy and so carried a point mutation. The mutants were designated Su297/A1, Su297/A10 and Su297/A12 and they required serine, isoleucine/valine and cytosine/uracil respectively.

Using the RDA plate technique (see general materials and methods) a methionine requiring auxotroph of G18 was isolated as a microcolony. The met G18 was designated G18-392.

Auxotrophs, and antibiotic resistant derivatives of W19 were provided by Dr. C. Ronson.

Isolation of antibiotic resistant mutants

Rifampicin and streptomycin resistant mutants of all strains used were isolated, as spontaneously resistant mutants, from stationary phase cultures of each parent by spreading 0.1 ml. of the culture on GSYC rif. or GSYC str. Mutants arose at a frequency of 10^{-6} - 10^{-8} depending on the strain and antibiotic. Doubly resistant mutants were isolated by selecting spontaneous mutants in two stages on the respective media. All mutants were purified on the selective media from single colonies. Finally, each mutant was tested for growth in the absence of the antibiotic to exclude the possibility that the mutant was antibiotic dependent.

Construction of R-factor containing strains.

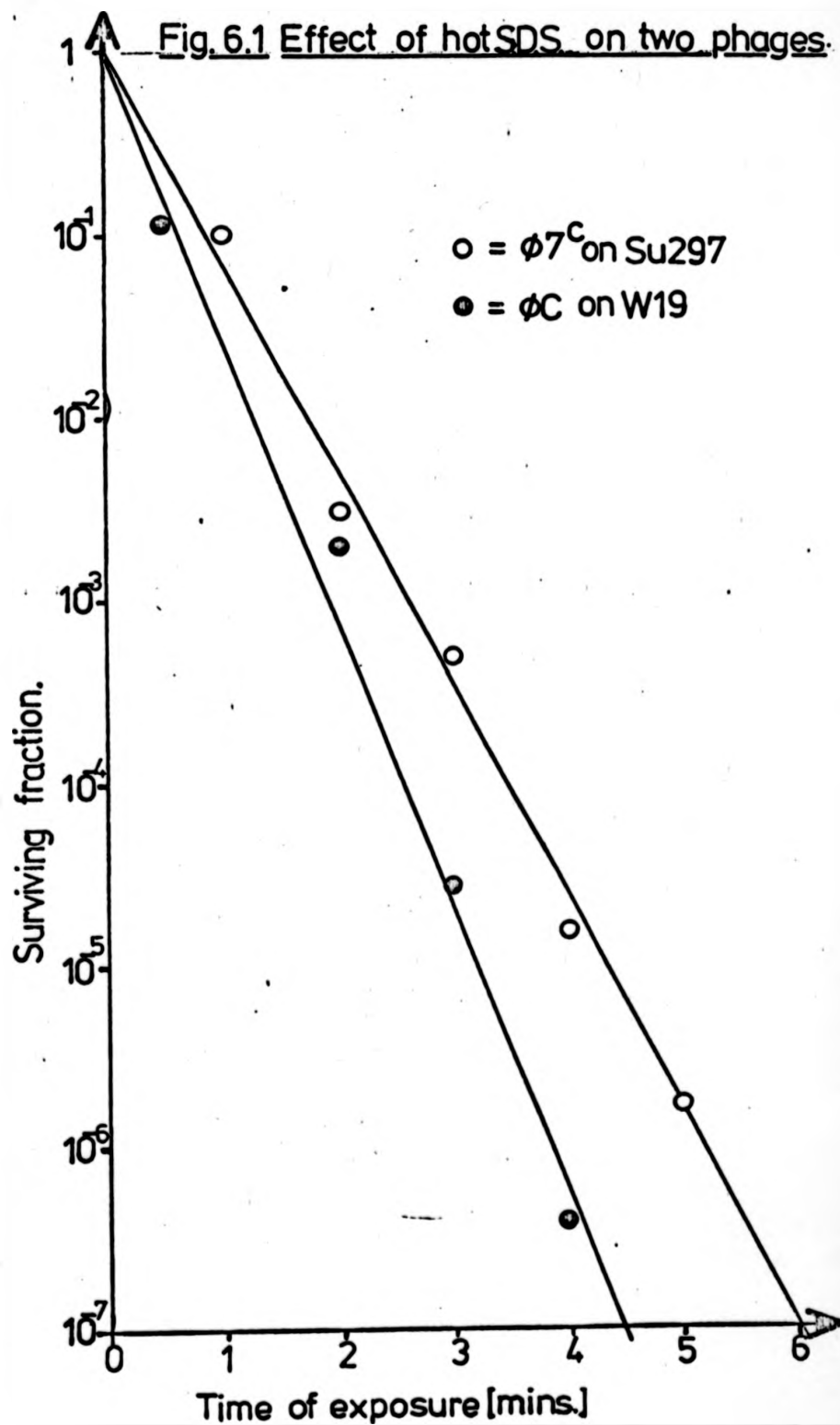
Donors and recipients were grown in GSYC broth to mid log phase of growth and then patched out onto GSYC agar plates. Three patches were made for each mating; a patch of mixed donor and recipient plus two controls. After overnight incubation at 30° the growth from each patch was streaked out on selective plates containing kanamycin (100 µg/ml) and either rifampicin (100 µg/ml) or streptomycin (200 µg/ml) as counter selective agent. Putative transconjugants were purified on the selective medium before testing for unselected markers; phage sensitivities, lysogeny and the ability to grow on nutrient agar.

Transduction method

Recipient cells were grown to log phase/stationary phase in GSYC broth at 30°. The cells were removed by centrifugation then washed twice with RDM before being resuspended in 1/10th original volume of RDM. This treatment generally yielded a final viable cell concentration of $5-10 \times 10^8$ c.f.u./ml. To this concentrated cell suspension phage was added to give a variety of m.o.i.¹⁸ and the phage/host mixture was left at room temperature for ten minutes to allow adsorption. Selective top agar (3ml) was then added to each tube and poured over selective bottom agar. Where antibiotic markers were selected the antibiotic was added, as a liquid overlay, to the top agar about 12 hrs. after plating. Controls were made for spontaneous reversion to prototrophy or mutation to antibiotic resistance and for sterility of the phage lysate. Where t.s. virulent phage (ϕC^{T616}) was used the reversion of the mutant to temperature insensitivity was quantified. Also, the viability of the recipient was quantified before infection.

Plates were examined, at daily intervals, for up to three weeks after plating. All colonies arising on assay and control plates were purified on the selective medium. Clones were then tested

Fig. 6.1 Effect of hot SDS on two phages.



for lysogeny; growth on NA plates; phage sensitivity and antibiogram reaction.

A variety of transductions were attempted (Table 6.1). Such transductions included the use of virulent phage (ϕC^{T616}) as well as the temperate phages $\phi S28$, $\phi 7$, $\phi 7^C$ and $\phi 8$.

Preparation of phage DNA

Phage lysates, of $\phi 7^C$ and ϕC , of 10^8 - 10^{10} p.f.u./ml. were prepared by plate lysate methods then filtered to remove debris. High titre lysates were then treated at 60°C in S.D.S. at 6.25%^v/v. Samples were removed and diluted into ice-cooled buffer, at intervals, for titration. From the inactivation curve (Fig.6.1) it was determined how long to treat the lysate to reduce the activity of the lysate to undetectable levels. The lysate, thus treated, was cooled on ice before dialysis against S.S.C. at 4°C to eliminate the S.D.S. The dialysed lysate was used as a crude DNA preparation.

Induction of competence

Cells of Su297 or W19 were grown to late log phase in LSB, GSYC or RDM before subculturing into fresh, prewarmed homologous medium. The cells were harvested, at various stages in the growth curve, for the induction of competence. For the induction of competence variations on the methods of Mandel and Higa (1970); Cohen *et.al.* (1972) and Cosloy and Oishi, (1973) were used. The basic method was as follows. The cultures were chilled on ice for 30 min. before centrifugation to harvest the cells. The cells were washed in $\frac{1}{2}$ original volume of ice-chilled 10mM NaCl and then harvested by centrifugation before being resuspended in $\frac{1}{2}$ original volume of ice-chilled 30mM CaCl_2 . The cells thus treated were held at 0°C for 20 min. after which they were harvested by centrifugation and resuspended in 1/10 original volume of ice-chilled 30 mM CaCl_2 .

Table 6.1

Transductions attempted in R. trifolii

DONOR	PHAGE VECTOR	RECIPIENT	SELECTED MARKER	SELECTION METHOD
W19SR(R ⁺)*	ØC ^{T616}	W19 <u>arg</u>	<u>arg</u> ⁺	GMA/30°
"	"	"	<u>str.</u> ^R <u>rif.</u> ^R	GSYC+rif+ str/30°
"	"	"	<u>tet</u> ^R	GSYC+tet. /30°
G18SR(R ⁺)*	ØS28	G18 <u>met</u>	<u>met</u> ⁺	GMA/30°
"	"	"	<u>str.</u> ^R <u>rif.</u> ^R	GSYC+rif+ str/30°
"	"	"	<u>tet.</u> ^R	GSYC+tet. /30°
Su297	Ø7. & Ø7 ^C	Su297A1.	<u>ser</u> ⁺	GMA/30°
Su297	Ø7, Ø7 ^C , Ø8	Su297A10	<u>ilv</u> ⁺	GMA/30°
Su297	Ø7, Ø7 ^C , Ø8	Su297A12	<u>pyr</u> ⁺	GMA/30°

* W19SR(R⁺) = W19str. rif (R68.45⁺)* G18SR(R⁺) = G18 str. rif (R68.45⁺)

Transfection method

Competent cells (0.2ml) were added to 0.1ml phage DNA and held at 0°C for 60 min. After this time the samples were subjected to a heat pulse of 42°C for 2 min. before being returned to 0°C for at least 60 min. before plating in top agar.

Experimental variations

In experiment one, cells were pregrown in three different media, namely LSB, GSYC and RDM and the cells were chilled and harvested at an O.D.₅₄₀ of 0.2. After being subjected to the method of competence induction as described above, samples were used in the transfection assay. In experiment two, cells were grown in GSYC and subjected to the competence induction treatment when the culture reached O.D.₅₄₀ of 0.1, 0.2, 0.3 and 0.4. In experiment three, cells were grown in GSYC to an O.D.₅₄₀ of 0.2 when they were subjected to the competence induction treatment. However the concentrations of NaCl used were 10mM, 20mM, 30mM and 40mM. In experiment four, cells were grown as in experiment three. NaCl was used at 10mM but instead of CaCl₂ at 30mM, MgCl₂ at 30mM was used. In experiment five, cells were grown as in experiment three. NaCl was used at 10mM and CaCl₂ was used at 30mM. However, the volumes of crude DNA solution used were 0.1ml., 0.2ml., 0.4ml and 1.0ml..

In all experiments, controls were made for the absence of viable phage in the DNA preparation; for the ability of the cells to form lawns after competence induction treatment; for the ability of phage to produce plaques on lawns of cells thus treated, and for the absence of plaques in the lawns of cells not subjected to phage DNA.

Results and Discussion

Transfection

In none of these experiments were plaques detected due to transfection. The phage DNA preparations were free from viable phage and cells used in the induction of competence were capable of forming lawns. Also there were no phage plaques detected in any lawns which were not exposed to phage DNA. However, the cells which had been subjected to the competence induction protocol were capable of plating $\phi 7^C$ or ϕC ; depending on the host strain used.

There could be many reasons why no transfection was detected. It was unknown whether the phage DNA samples contained intact or degraded DNA. It has been demonstrated that the physical state of the DNA can affect the transfection efficiency (Benzinger, 1978). It was not known if the phage DNA was capable of binding to the treated cells or whether or not the DNA was actually taken up by the cells. Hence, if the phage DNA could not bind to the cells; or if the physical state of the DNA was not conducive to transfection, then no transfection could occur. Alternatively, the phage DNA may have been intact but the cells were not competent. Finally, the DNA may have been chemically degraded either inside or outside the cell. It is well documented that a whole variety of conditions need to be satisfied before most species of bacteria can be transformed or transfected (e.g. see Benzing, 1978). Why the rhizobia should be so refractory to transfection or transformation is a total mystery. Recently it has proved possible to transform Pseudomonas (Mylroie, et.al., 1977; Gantotti et.al., 1979) and Agrobacterium (Holsters et.al., 1978), although the efficiencies of transformation have not been high. So it may be that Rhizobium requires particularly stringent conditions for transfection or transformation to be effective. Conversations with Dr. J. Beringer

and Dr. A. Johnston (John Innes Institute, Norwich) revealed that extensive attempts at transforming R. leguminosarum were also totally fruitless. The fact that there have been no unequivocal demonstrations, of rhizobial transformation, in the literature to date makes it seem likely that either special conditions will have to be provided to effect transformation in this genus or else the rhizobia are totally resistant to transformation.

Construction of R⁺ strains

R68.45 was transferred from E.coli 1230 into rhizobial recipients in plate matings, counter-selecting the donor with rifampicin or streptomycin. RP1 was transferred from P.aeruginosa into rhizobia, using identical methods. Although rifampicin counterselection was not very efficient for Pseudomonas it proved possible to isolate pure rhizobial trans-conjugants by standard colony purification methods. Exconjugants were examined for their antibiograms; phage sensitivities; phage release and ability to grow on NA. The results are presented in Tables 6.2 to 6.6.

The data in Table 6.2 show that R68.45 containing Su297, Su298 and Nul8 have similar antibiogram reactions. The parental strains are hypersensitive to tetracycline whereas the R⁺ derivatives show a far higher resistance. Similarly the R⁺ derivatives become more resistant to carbenicillin, neomycin, penicillin, kanamycin and cephalothin, than the parents. Correspondingly, markers which are not carried by R68.45 e.g. streptomycin or nalidixic acid resistance are absent from parents and R⁺ derivatives. From such results it is reasonable to conclude that R68.45 was transferred into Su297, Su298 and Nul8. The frequency of R-factor transfer into these strains was not determined but transconjugants were obtained with ease using the plate mating methods. Derivatives of Su297R⁺, which were lysogenic for $\phi 7$, $\phi 8$ or $\phi 7^C$, reacted identically in the antibiogram tests, to the non-lysogenic R⁺ derivatives (data not presented).

Table 6.2

Exconjugant antibiograms

STRAIN	Te	Py	N	Pn	K	S	Rd	Kf.	Na	Ct.
Su297	21	14	3	8	3	3	15	13	3	0
Su298	20	16	3	10	4	4	16	16	8	1
Nu18	20	16	3	8	4	5	17	17	8	0
Su297R ⁺	3	4	0	0	0	0	15	4	4	0
Su297R ⁺	3	4	1	0	0	1	0	5	3	0
Nu18R ⁺ S.	3	6	1	2	0	0	14	10	5	0
Su298R ⁺ R.	3	6	1	2	0	2	0	5	3	0
<u>E.coli</u> 1230	1	0	0	0	0	7	9	0	11	0

Inhibition zones were measured in mm.

Su297R⁺ = Su297A10 str (R68.45)

Su297R⁺ = Su297A10 rif (R.68.45)

Nu18R⁺S = Nu18 str (R68.45)

Su298R⁺R = Su298 rif (R.68.45)

Table 6.3

Phage sensitivities

STRAIN	PHAGE			
	Øi.Su297	Ø7.Su297	Ø8.Su297	Ø8.Su298
Su297	-	T.L.	T.L.	T.L.
Su297(7)	-	-	-	-
Su297(7)R ⁺	-	-	-	-
Su297R ⁺	-	T.L.	T.L.	T.L.
Su298	S.T.L.	S.T.L.	S.T.L.	S.T.L.
Su298R ⁺	S.T.L.	S.T.L.	S.T.L.	S.T.L.
Nul8	Øi.Nul8	Ø7.Nul8	Ø8.Nul8	
	T.L.	T.L.	T.L.	
	T.L.	T.L.	T.L.	

E.coli 1230 was resistant to all rhizobiophages used in this study.

T.L. = turbid lysis
 S.T.L. = slightly turbid lysis
 - = resistant

Table 6.4Phage release

SUPERNATANT

STRAIN	Su297R ⁺	Su298R ⁺	Nu18R ⁺
Su297	-	-	-
Su297R ⁺	-	-	-
Su298	T.L.	-	-
Su298R ⁺	T.L.	-	-
Nu18	T.L.	-	-
Nu18R ⁺	T.L.	-	-

T.L. = turbid lysis

- = resistant

Table 6.5Antibiograms

STRAIN	ANTIBIOTIC													
	Va	Pn	Sxt	Na	Pb	Nv	S	SH	K	N	E	C	PY	Te
G18RR	7	10	0	3	2	0	5	4	2	3	0	3	15	20
G18R ⁺ RR	8	3	0	3	2	0	5	4	0	0	0	3	2	2
<u>E.coli</u> 1230	0	0	8	0	0	0	4	0	0	0	0	8	0	0

G18RR = G18 rif.

G18R⁺RR = G18 rif. R68.45

Table 6.6Phage sensitivity

PHAGE

STRAIN	ØC.W19	ØC.G18	ØS28.G18	Ø7.Su297
G18RR	T.L.	C.L.	T.L.	-
G18R ⁺ RR	T.L.	C.L.	T.L.	-

T.L. = turbid lysis

C.L. = clear lysis

- = resistant

Therefore lysogenisation with any of these phages does not affect the antibiotic sensitivity of the host although it does affect the phage sensitivity and somatic antigens, (Barnet and Vincent, 1970).

The data in Tables 6.3 and 6.4 show that the presence of the R-factor does not affect the phage sensitivities of the strains or the release of resident phage into the supernatant. So R68.45 produced no observable effects on the phage adsorption or replication aspects of these strains. Neither does R68.45 affect the lysogenic stability of Su297 lysogens.

The R^+ derivatives of Su297, Su298 and Nul8 were spot tested with ϕ PRR1. The lysate was 10^{10} p.f.u./ml. as titred on P.aeruginosa PAT 904 rev 1. However the R^+ rhizobia and the E.coli 1230 strains were resistant to ϕ PRR1. When $RP1^+$ derivatives of Su297, Su298 and Nul8 were constructed and tested as before they reacted in an identical fashion to the R68.45 derivatives (data not presented). When such $RP1^+$ derivatives were tested for ϕ PRR1 sensitivity, they proved to be resistant. The $RP1$ was transferred from Su297 $RP1^+$ rif to Su298 str and so there was no correlation between ϕ PRR1 resistance and lack of conjugal transfer ability in these strains. Hence the reason for ϕ PRR1 resistance in these $RP1^+$ and R68.45 $^+$ rhizobia is unknown. However analogues of this phenomenon exist in other genera (e.g. Mindich et.al., 1976; Alexander and Jollick, 1977).

The data in Table 6.5 show that R68.45 was transferred into G18RR. As found for Su297 derivatives, the presence of R68.45 did not affect the susceptibility of G18RR to the phages which plated on the R^- parent. (Table 6.6).

As a final test, all of these R^- and R^+ derivatives were streaked out on NA at 30°. Although the E.coli and P.aeruginosa donors grew well on NA, none of the rhizobial strains grew on this medium.

Transduction using ϕC^{T616} ; the conditional lethal virulent phage

In the experiments involving ϕC^{T616} no transductants were ever detected for either arg⁺, str^R, rif^R or tet^R markers. These markers were chosen because of their low revertability (met, $\sim 10^{-8}$) or their extremely low spontaneous mutation frequency (tet^R, $< 10^{-9}$). The markers str^R and rif^R cannot be adequately separated by conjugational analysis (Beringer and Hopwood, 1976) and so it seemed reasonable that they may be co-transducible. Indeed this has very recently been demonstrated to be so (Buchanan-Wollaston, 1979). Why it was impossible to detect transduction in these experiments cannot be said with certainty. However, in control assays for reversion of the T616 mutation to temperature insensitivity it was found that it reverted with a frequency of 10^{-6} - 10^{-7} . Therefore, perhaps even if "transductants" were generated in any of these assays they may have been killed by reverted phage as indeed occurs with the T1 system (Drexler and Kylberg, 1975). Hence a mutant phage with a lower reversion frequency would have been more useful here; or a double mutant.

Because of this severe limitation on the level at which transduction could have been detected using ϕC^{T616} it is impossible to say whether or not this conditional lethal phage could be used for transduction in R.trifolii. Perhaps the use of the restrictive temperature in combination with an ultra violet inactivation treatment (Buchanan-Wollaston, 1979) would prove effective.

The DNA of the virulent, generalised transducing phage, T1, is not circularly permuted and T1 packages its DNA by a self determined mechanism

which may be important for some aspects of the T1 transducing ability (Drexler, 1977). Information of this nature is not available for ϕC although this phage does have a high proportion

of repeated base sequences (Atkins and Avery, 1974). There is no evidence, then, that ϕC has the physical capability to generate transducing particles.

Transduction using $\phi S28$

As with ϕC^{T616} , no transductants were ever detected in an assay using $\phi S28$. The phage $\phi S28$ is a temperate phage in G18 (this study) and so less phage induced killing of possible transductants would be expected. It is possible that $\phi S28$ can transduce but may be capable only of restricted transduction like λ (Hayes, 1968). In such a situation, transduction of markers which are not adjacent to the $\phi S28$ attachment site would not be detected. It is also possible that $\phi S28$ can transduce the antibiotic resistance markers but that inadequate time was allowed for the injection, recombination and expression of the antibiotic resistance determinants. However, this seems unlikely. As with ϕC^{T616} a range of multiplicities of infection were used in these assays; ranging from 0.01 to 10 and so it also appears unlikely that transduction was not detected due to the selection of an inappropriate m.o.i.. Consequently, as with ϕC^{T616} , there is no reason to believe that $\phi S28$ can act as a transducing vector for rhizobial DNA.

Transduction using $\phi 7$, $\phi 7^C$ and $\phi 8$

The data from several transductions (designated experiments A-F) are presented in Table 6.7. In these experiments all lysates were free of bacterial contamination. Tests for reversion frequency were conducted in duplicate or triplicate using the same number of cells used in transduction assay plates, so that a direct comparison could be made between colony numbers. In general some non-selected marker was used in the recipient strains by which identification of any putative transductants could be achieved. This marker was either an antibiotic resistance marker or a phage sensitivity

Table 6.7

Results of transduction assays

EXPERIMENT	PHAGE	METHOD OF PREPARATION	PHAGE TITRE	RECIPIENT	VIABLE CELL COUNT	COLONIES PER PLATE	
						+PHAGE	-PHAGE
A.	Ø7.Su297	confluent lysis	4.0×10^8	Su297A1	1.6×10^8	120	138
				Su297A10	3.0×10^8	129	0
				Su297A12	5.0×10^8	110	1
A.	Ø7 ^C .Su297	confluent lysis	6.0×10^7	Su297A1	1.6×10^8	160	138
				Su297A10	3.0×10^8	0	0
				Su297A12	5.0×10^8	1	1
B.	Ø7.Su297	MC-induced	1.5×10^8	Su297A1	5.0×10^8	205	410
				Su297A10	4.0×10^8	3	2
				Su297A12	8.0×10^8	106 large+ 150 small	7 large+ 160 small
B.	Ø7 ^C .Su297	MC-induced	9.8×10^8	Su297A1	5.0×10^8	20	410
				Su297A10	4.0×10^8	2	2
				Su297A12	8.0×10^8	68 large/ small	7 large + 160 small
C.	Ø7.Su297	MC-induced	4.5×10^8	Su297A10S	4.7×10^8	7	0
				Su297A12	3.0×10^8	20 large+ 80 small	13 large + 600 small

Table 6.7 (Contd.)

EXPERIMENT	PHAGE	METHOD OF PREPARATION	PHAGE TITRE	RECIPIENT	m.o.i.	COLONIES PER PLATE	
						+PHAGE	-PHAGE
D.	Ø7.Su297	confluent lysis	9.0×10^8	Su297A10S	8	2	0
					3	0	
					1.5	0	
					0.8	0	
D.				Su297A12S	11	38	1
					4.5	28	
					2	10	
					1	0	
					0.2	0	
E.	Ø8.Su297	confluent lysis	2.2×10^9	Su297A10S	25	2	1
					5	0	
E.				Su297A12S	16	24	3
					3	3	
F.	Ø7.Su297	confluent lysis	1.0×10^9	Su297A12	20	21	2
	Ø7.Su297 A12	confluent lysis	6.0×10^8	Su297A12	30	0	2
					17	0	2

determinant. Finally, to exclude the possibility that the phage itself was mutagenic, assays were done using lysates prepared on the homologous host to the transductional recipient e.g. experiment F.

In experiment A the wild type and clear plaque mutant of $\phi 7$ were used to try to transduce three auxotrophs of Su297 to prototrophy. The Su297A1 (serine-mutant) reverted with such frequency that it proved to be of little utility in such an assay. Using $\phi 7^C$ no apparent increase in colony number, over the control value, was achieved. Both the A10 and A12 mutants yielded low numbers of revertants and therefore were good mutants with which to screen for transduction. The $\phi 7^C$ lysate was of low titre and therefore the numbers of potential transducing particles would have been low in such a lysate.

Using $\phi 7$.Su297 as the lysate there was no stimulation over the number of colonies found in the Su297A1 reversion assay. However, for the Su297A10 and Su297A12 assay plates, there were more colonies in the presence of the phage than on the control plates. In both transduction assays the colonies were irregular and often surrounded by micro-colonies. This was probably due to cross-feeding from the larger colonies.

Because the phage lysate itself was free from bacterial contamination, and because the number of revertants was low, it seems that the phage lysate was responsible for the stimulation in reversion frequency. There could be several explanations for this result. It is possible that the stimulation was due to transduction or transformation. Alternatively, the phage, or something else in the lysate, could be mutagenic, thereby enhancing reversion. Finally, it is possible that some of the tubes used in the assay were contaminated. If the "lysate-stimulated reversion" (L.S.R.) was due to transduction it would be necessary to explain the significant

difference in effect due to the clear-plaque mutant compared with that of the wild type phage. It is possible to explain such an effect by assuming that $\phi 7^C$ would kill more potential transductants, than the $\phi 7$ wild-type. Alternatively, the low titre of the $\phi 7^C$ lysate, compared with the $\phi 7$ lysate, would perhaps mean that there would be less potential transducing particles in the former lysate.

Because this was the first attempt at transduction in this system, and because the colonies which arose, although slow growing, were irregular in appearance, it was considered most likely that the L.S.R. was due to contamination. Unfortunately, for this reason, these colonies were not examined further, although retrospectively this was an error. However, the experiment was repeated. It seemed reasonable that if the L.S.R. had been due to transduction that the transduction was of a generalised form since two markers were "transduced".

The data from experiment B show that lysates produced by M.C. induction of lysogens were also used. Both lysates had been dialysed before use in an attempt to eliminate the mitomycin. This was done because residual MC could have enhanced the reversion of the auxotrophs to prototrophy.

In contrast with the data from experiment A, the $\phi 7^C$ titre was higher than that of the $\phi 7$ parent. Again it was obvious that Su297A1 reverted at too high a frequency to be of use in these transductional assay systems. Nevertheless, with both $\phi 7$ and $\phi 7^C$ lysates the number of colonies present on the assay plates of Su297A1 were actually less than on the reversion control plates. This suggests that the phage was responsible for killing of some revertants and, by the same argument, any "transductants" which may have been generated. With $\phi 7$ the survival was only 49% of the revertant control plate level; with $\phi 7^C$ the survival was 5% of the revertant control level. In

experiment A the number of colonies of Su297A1 was about the same with or without $\phi 7$ (85%) but there may have been a slight stimulation in the presence of $\phi 7^C$. However, the m.o.i. with $\phi 7^C$ was only about 10% of that with $\phi 7$. Hence there may have been more killing by the phage in the $\phi 7$ lysate compared with the killing effect of the $\phi 7^C$ lysate.

In experiment B no L.S.R. was noted, in the presence of $\phi 7^C$, with Su297A1 or Su297A10. With Su297A12, 68 irregular, medium sized colonies arose in the presence of $\phi 7^C$. This compares with a control plate reversion level of 7 colonies. The background of micro-colonies on the revertant plates was presumed to be most likely the result of cross-feeding, although on this occasion a similar effect was not noted on the transduction assay plate. This may have been due to phage killing of the cross-fed colonies. By this logic the colonies which did grow up would be expected to be lysogens, phage resistant or fortunate to have grown in an area of low phage density.

In the presence of phage, Su297A10 yielded the same number of colonies as in the reversion control plates. Bearing in mind the likely phage killing effect it would be theoretically possible that a proportion of these were "transductants" although there is no evidence that this was so. Hence there was no L.S.R. in experiment B. This result is in contrast to the results from experiment A. Yet with Su297A12 in the presence of $\phi 7$ or $\phi 7^C$ there was a stimulation of colony number over the reversion frequency, even although there was a relatively high number of colonies on the reversion plates. If the small colonies were due to crossfeeding effects then the relative increase in colony number due to the phage lysate was 15 times. Why this level of stimulation should occur with the Su297A12 mutant on this occasion but not with the Su297A10 mutant, as in experiment A, is a mystery. Perhaps the m.o.i. is of crucial

importance. It is known that certain genes can be transduced more frequently than others in E.coli, using P1 (Masters and Broda, 1971). It is also possible that the enhanced growth on the plates with the phage lysate was due to nutrients carried over from the lysate. However if this had been so one would have expected to have found a slight background lawn rather than isolated colonies and a similar level of "reversion" of all three auxotrophs. At this stage it was suspected that some form of transductional phenomenon was occurring.

The results from experiment C show no reversion of Su297A10 to prototrophy. However, in the presence of Ø7, seven colonies arose. With Su297A12 in the presence of Ø7, the background small colonies were reduced in number compared with the numbers obtained on the control plates. Nevertheless, the numbers of large colonies did increase slightly in the presence of the phage. However it was impossible to say if this result was significant or not. Because the Su297A10 assay had produced such "clean" results these seven colonies were purified and examined further (see page 241).

Albeit variable, this L.S.R. effect was repeatable to some extent and it was important to know if it was only attributable to the lysate used. A fresh lysate of 9×10^8 p.f.u./ml. was used to repeat the experiments. Using this lysate with Su297A10S, two colonies arose at the 5x lysate concentration although no colonies arose on the reversion control plates, (experiment D). However, the phage lysate sterility control plate only contained 1/6th of the lysate volume as in the 5x assay plates. If bacterial contamination was present in the lysate at a very low level this could have accounted for the observed differences.

With Su297A12S and the phage at the neat and 5x concentrations there was a L.S.R. effect but at lower m.o.i. no L.S.R. effect was

noted. This suggests that the L.S.R. effect is very dependent on the dilution of the lysate, but yields no information about the component of the lysate responsible for the effect. Some of the Su297A12S clones from the assay plates were purified and examined further (see page 244).

In experiment E, Ø8.Su297 was used as the lysate, rather than Ø7. Since both of these phages are closely related (see chapter V) it is not unreasonable to think that Ø8 lysates could cause a L.S.R. effect like Ø7. Again, using Su297A10S, no obvious stimulation of reversion occurred with the Ø8 lysate, even at high m.o.i.. However, using Su297A12S, a stimulation in the reversion of 8 x the control value was noted, with Ø8 at the 5x concentration. This time a 5x control phage lysate sterility plate was sterile. Consequently, a Ø8 lysate could give the L.S.R. effect because these colonies were unlikely to be due to contamination. A few of these colonies were purified from the assay plates and examined further. (see page 245).

Experiment F was designed to find out if the genetic background of the host on which the lysate was made was important for the L.S.R. effect. The data show that the L.S.R. effect occurred with high Ø7.Su297 concentration. However, even at 10x concentration the Ø7.Su297A12 lysate did not yield any L.S.R. effect on the homologous host. In fact not even one revertant colony was noted in the phage assay plates so some phage killing of the revertants had probably occurred. It seems likely, therefore, that the L.S.R. effect is dependent on the genetic background of the host on which the phage lysate had been made. Such a result is compatible with a transduction or transformation model for the genesis of the L.S.R. effect. The data from experiment F suggest that the phage itself cannot be mutagenic.

Insufficient time was available in this study to repeat these assays or to find out the effects of DNA-ase, or anti-phage antiserum, on the L.S.R. effect. However, ϕ i.Su297 lysates (5×10^8 p.f.u./ml) yielded no L.S.R. with Su297A12S suggesting that transformation was not the cause of the effect.

Hence, experiments A-F show that, although variable, this effect can be repeated and can be induced by ϕ 7, ϕ 8 and perhaps ϕ 7^C. There are several possible explanations for the L.S.R. effect.

- 1) The phage or some other component of the lysate acts as a mutagen thereby increasing the reversion frequency. This possibility is probably excluded by the results from experiment F.
- 2) The lysate may carry over nutrients which allow slight growth of the auxotrophs and so the observed reversion frequency is stimulated under these conditions. This is unlikely because, as stated before, one would expect a slight background lawn of growth in the assay plates rather than isolated colonies. Also, one would expect that ϕ 7.Su297A12 would be as capable as ϕ 7.Su297 to induce L.S.R. on Su297A12 if this reason were correct. This did not occur.
- 3) The L.S.R. effect may be due to transformation. Although this possibility cannot be excluded on the basis of the weak evidence presented in this study, it does seem unlikely since ϕ i.Su297 lysates do not yield a L.S.R. effect.
- 4) The effect may be due to transduction. By analogy with the classical models of transduction, several possibilities could exist.

If the effect was due to generalised transduction then the transductants could be of two major types. The first type would be non-lysogenic; would not release ϕ 7 (or ϕ 8); and would be ϕ 7 sensitive. The second type could be lysogenic for ϕ 7 where the

"transductant" had been superinfected by a normal phage particle and lysogeny had been established. In such a situation the transductant would yield the classical phenotype of a ϕ 7 lysogen of the prototrophic parental Su297.

If the effect was due to restricted transduction the "transductants" could be also of two major types. The first type would be prototrophic due to lysogenisation by a plaque forming phage carrying part phage and part host DNA. The second type would be prototrophic due to infection by a defective (non-plaque forming) phage carrying sufficient host DNA to eliminate phage genes essential for lytic development and packaging of nucleic acid in the host. Transductants of the former type could yield high frequency transducing (h.f.t.) lysates as opposed to the low frequency transducing (l.f.t.) lysates from which they were derived. Of course, one would not expect to be able to generate restricted transducing particles from lysates produced by the confluent lysis method. This is because the transducing particles produced in restricted transduction are generated by chance errors in the fidelity of excision of the prophage DNA from the host genome, thereby allowing small sequences of contiguous host DNA to be packaged along with the remainder of the phage DNA (e.g. see Chapter 21 of Hayes, 1968).

"Transductant" analysis

To determine which, if any, of the above possibilities was the most likely it was essential to characterise the putative transductants more fully. Hence representative colonies were purified then tested for phage release, phage sensitivity; antibiogram reaction; sensitivity to oxid nutrient agar and, in some cases, the ability of supernatants to recomplement the corresponding mutant at high frequency.

a) Analysis of putative transductants from experiment C

The data in Tables 6,8 to 6,12 show that each of the putative transductants (p.t.'s.) from experiment C (see Table 6.11) reacted in an identical fashion. Tra-1 to Tra-7 were all resistant to all of the phages tested, including some phages which plated on the Su297 present. Either this could have been due to phage conversion which is known to occur in this system (Barnet and Vincent, 1970; this study) or multiply resistant mutants had been selected in the assay plates. If the latter is true then spontaneous mutation to resistance to one phage leads to total cross-resistance to the other phages, implying a common cell surface receptor site for those phages. However, although the spontaneous mutation frequency of Su297A10 to $\phi 7$ resistance is unknown, one would not expect it to be radically different from the spontaneous mutation frequencies for other markers e.g. rif^R and str^R of 10^{-6} - 10^{-8} (Although spontaneous mutation to tet^R does not seem to occur at this frequency i.e. $<10^{-9}$). Because the reversion rate of the A10 mutant to prototrophy is about 10^{-8} then the probability of spontaneous mutation to prototrophy and $\phi 7$ resistance, simultaneously, would be of the order of 10^{-14} . This means that, unless mutation to $\phi 7$ resistance confers prototrophy upon the A10 mutant, or vice-versa, the possibility that this has occurred here is very low indeed. Because Su297A10 revertants to prototrophy are still $\phi 7$ sensitive this latter possibility can be excluded. One would expect these p.t.'s. to be $\phi 7$ lysogens, possibly due to double infection with a generalised transducing phage and a plaque forming parent. Because the lysate used was produced by MC induction it is not impossible that the p.t.'s. arose from plaque forming or defective transducing particles which carried both phage gene(s) for lysogenic conversion and the wild type A10 allele.

From Table 6.8 it can be seen that all p.t.'s reacted in the same fashion in that none released a phage which plated on Su297A10.

Table 6.8

Phage sensitivities of p.t.'s from experiment C

TEST STRAIN	PHAGE				
	Ø7.Su297	Ø8.Su297	Øi.Su297	Ø7 ^C .Su297	H.S.
TRA-1	-	-	-	-	-
TRA-2	-	-	-	-	-
TRA-3	-	-	-	-	-
TRA-4	-	-	-	-	-
TRA-5	-	-	-	-	-
TRA-6	-	-	-	-	-
TRA-7	-	-	-	-	-
Su297	T.L.	T.L.	T.L.	T.L.	-
Su297A10S	T.L.	T.L.	T.L.	T.L.	-

- = negative

T.L. = turbid lysis

H.S. = homologous supernatant

Table 6.9 Phage release from p.t.'s from experiment C

SUPERNATANT	TESTER RECIPIENT		
	Su297A10	Su298	Nu18
TRA-1	-	S.T.L.	T.L.
TRA-2	-	S.T.L.	T.L.
TRA-3	-	S.T.L.	T.L.
TRA-4	-	S.T.L.	T.L.
TRA-5	-	S.T.L.	T.L.
TRA-6	-	S.T.L.	T.L.
TRA-7	-	S.T.L.	T.L.
Ø7.Su297	T.L.	T.L.	T.L.
Ø8.Su298	T.L.	T.L.	T.L.
Ø1.Su297	-	T.L.	T.L.
Su297A10S(7)	T.L.	S.T.L.	T.L.

S.T.L. = slight turbid lysis

T.L. = turbid lysis

- = negative

Table 6,10

MC-inducibility of p.t.'s from experiment C

p.t.	TITRE ON Su297 (p.f.u./ml)	TITRE ON Nu18 (p.f.u./ml)		% increase
	+m.c.	+ MC	- MC	
TRA-1	N.D.	4×10^5	5×10^7	125
TRA-2	N.D.	3×10^5	5×10^7	167
TRA-3	N.D.	4×10^5	6×10^7	150
TRA-4	N.D.	5×10^5	7×10^7	140
TRA-5	N.D.	2×10^5	3×10^7	150
TRA-6	N.D.	5×10^5	8×10^7	160
TRA-7	N.D.	2×10^5	3×10^7	150

N.D. = none detected

Table 6.11 Results of complementation assays of p.t. supernatants on Su297A10

p.t.	MC-induced culture supernatant	non-induced culture supernatant
TRA-1	-	-
TRA-2	-	-
TRA-3	-	-
TRA-4	-	-
TRA-5	-	-
TRA-6	-	-
TRA-7	-	-

- = negative

Table 6.12

Antibiograms of p.t.'s from experiment C

p.t.	ANTIBIOTIC			
	TE(50)	PY(100)	K(50)	S(25)
TRA-1	20	19	1	0
TRA-2	20	19	0	0
TRA-3	19	19	0	0
TRA-4	20	18	0	0
TRA-5	19	19	1	0
TRA-6	20	19	0	0
TRA-7	19	19	0	0
Su297A10S	20	19	1	0
Su297	19	19	5	5

Inhibition zones are measured in mm.

Either the p.t.'s are not $\phi 7$ lysogens; they release defective $\phi 7$ or they cannot release $\phi 7$ for some reason. For both lysogenic conversion and transduction to occur simultaneously from one infection one has to predict a model whereby bacterial and phage DNA are ligated in the same nucleocapsid. Such a model is difficult to imagine except by a system similar to λ or $\phi 80$ mediated transduction in which $\phi 7$ would have an integration site close to the A10 marker. On excision, defective particles would be assembled in which phage gene(s) for conversion would be present but all of the genes needed for normal lytic development were not. If, on integration into the Su297A10S genome, prototrophy results so will phage conversion. However, although plaque forming phage may not be able to be made by such an integrated unit, assuming no "helper" action by the related, hetero-immune resident ϕi , lysates could be formed which, if assisted by helper phage could transduce phage resistance and prototrophy, simultaneously, at high frequency. Perhaps these p.t.'s could have been produced by a mechanism similar to that of the transductants produced by $\phi 16-3dc$ particles, in the system studied recently in R.meliloti (Svab et.al., 1978). In such a system it is proposed that the defective particles, carrying the bacterial DNA, are capable of integrating into the chromosome but are not capable of excising from the chromosome. Hence the p.t.'s would express prototrophy and phage resistance but would not release infectious phage.

The data from Table 6.10 show that all of the TRA p.t.'s are MC-inducible and the release of ϕi , the resident prophage, is increased by ~150% compared with the spontaneous release level. However, no $\phi 7$ was detected on Su297, even after MC induction. This result excludes the possibility that an integrated plaque forming transducing phage could be present but be so stably integrated that it is not released.

From the complementation data in Table 6.11 it can be seen that no re-complementation of Su297A10S occurred with the induced or uninduced culture supernatants, even in the presence of helper phage.

The p.t.'s from experiment C cannot be contaminants either, because they release ϕ_i (Table 6.9); they carry the streptomycin resistance marker of the parent; they have identical general anti-biogram patterns to the parent (Table 6.12) and they all fail to grow on NA.

It is difficult then to devise one catholic model to explain the biogenesis of such p.t.'s, although several hypothesis can be considered.

- (1) If, say, within the population of cells in the assay some were ϕ_7^R then one would not expect them to be "transduced". This is because, although Plkc lysogens can be transduced by Plkc (M. Masters, pers.comm.), ϕ_7 lysogens of Su297 express multiple phage resistance by preventing the adsorption of related phages (Barnet and Vincent, 1970). Therefore, one has to invoke a model whereby mutation to ϕ_7^R arises post infection by the transducing particle. However it would be expected that the probability of lysogenisation by ϕ_7 would be far greater than that of ϕ_7 resistance occurring spontaneously. Consequently lysogens rather than resistant mutants would be expected to predominate.
- (2) Perhaps the p.t.'s are really due to transformation and the gene for A10 complementation maps very close to the genes needed for conversion in the resident prophage. So, in this case, co-transformation of both would occur with significant frequency. In such a case no phage release would be possible, due to the incomplete transfer of phage DNA, and multiple phage resistance could result. However, bearing in mind that there is poor

evidence for transformation in R.trifolii this seems unlikely. Also, one might expect that ϕ i.Su297 lysates would at least be capable of generating p.t.'s with Su297A10 even if they did not show phenotypic multiple phage resistance.

- (3) If these 7 colonies had just been due to reversion of the A10 strain to prototrophy one would expect ϕ 7 lysogens to arise rather than multiple resistant mutants.
- (4) The p.t.'s could have been contaminants but the data in Tables 6.9 and 6.12 disprove this possibility. Correspondingly if they had been Su297 contaminants from the ϕ 7 lysate they would not be str^R and would probably be ϕ 7 lysogens.
- (5) Perhaps low MC-levels in the lysate enhanced the mutagenesis rate thereby increasing the reversion frequency. Under such circumstances again, however, one would expect to find ϕ 7 lysogens rather than prototrophic; multiply resistant mutants.

Therefore it seems unlikely that there is one immediately obvious explanation for these results. Inadequate data are available regarding the molecular biology or genetics of the ϕ 7/Su297 system on which it may be possible to predict a molecular model which would adequately describe this phenomenon. It appears that no analogy exists for this effect in the well defined phage/host systems e.g. λ , ϕ 80 or P1 E.coli or P22 in S.typhimurium. Some artefact of the technique may also be responsible for the effect and the reason for this may be beyond the reach of this rather crude analysis (see later).

b) Analysis of putative transductants from experiment D

The antibiogram data (Table 6.13) show that all of the p.t.'s in this NTRA series 1-20 carry the markers of the Su297A 12S parent. Also they failed to grow on NA and are therefore not likely to be non-rhizobial contaminants. Because they carry the str^R marker of

Table 6.13 Antibiograms of p.t.'s from experiment D

STRAIN	ANTIBIOTIC						
	S(25)	K(30)	N(30)	E(50)	C(50)	TE(50)	PY(100)
NTRA-1	0	0	0	0	7	18	17
NTRA-2	0	0	0	0	8	18	18
NTRA-3	0	0	0	0	7	18	18
NTRA-4	0	0	0	0	9	18	18
NTRA-5	0	0	0	0	7	18	18
NTRA-6	0	0	0	0	7	17	18
NTRA-7	0	0	0	0	8	18	17
NTRA-8	0	0	0	0	7	18	18
NTRA-9	0	0	0	0	9	18	18
NTRA-10	-	-	-	-	-	-	-
NTRA-11	0	0	0	0	7	18	17
NTRA-12	0	0	0	0	8	17	17
NTRA-13	0	0	0	0	8	18	18
NTRA-14	0	0	0	0	7	18	18
NTRA-15	0	0	0	0	8	18	18
NTRA-16	0	0	0	0	7	17	18
NTRA-17	0	0	0	0	7	18	18
NTRA-18	0	0	0	0	8	18	17
NTRA-19	0	0	0	0	8	18	18
NTRA-20	0	0	0	0	8	18	18
Su297	2	3	3	0	8	18	18
Su297 A12S	0	0	0	0	7	18	18

Inhibition zones are in mm.
 - = no growth at all

Table 6.14

Phage sensitivities of p.t.'s from experiment D

STRAIN	LYSATE			
	Øi.Nu18	Ø7.Su297	Ø8.Su297	H.S.
NTRA-1	C.L.	S.T.L.	T.L.	-
NTRA-2	-	-	-	-
NTRA-3	-	C.L.	I.C.P.	-
NTRA-4	-	C.L.	I.C.P.	-
NTRA-5	T.L.	-	T.L.	-
NTRA-6	T.L.	-	T.L.	-
NTRA-7*	-	C.L.	?I.C.P.	-
NTRA-8	-	C.L.	I.C.P.	-
NTRA-9*	-	C.L.	?I.C.P.	-
NTRA-10	O	O	O	O
NTRA-11	-	C.L.	I.C.P.	-
NTRA-12*	-	C.L.	I.C.P.	-
NTRA-13*	-	C.L.	?I.C.P.	-
NTRA-14*	-	?C.L.	?	-
NTRA-15*	-	?C.L.	?	-
NTRA-16*	-	C.L.	I.C.P.	-
NTRA-17*	-	C.L.	I.C.P.	-
NTRA-18*	-	C.L.	I.C.P.	-
NTRA-19	C.L./T.L.	-	T.L.	-
NTRA-20	-	C.L.	I.C.P.	-
Su297	-	T.L.	T.L.	-

- * = autplaques over all of lawn
 T.L. = turbid lysis
 C.L. = clear lysis
 O = no growth at all
 - = resistant
 S.T.L. = slight turbid lysis
 I.C.P. = isolated clear plaques
 ?C.L. = probably clear lysis but background autplaques are too numerous to tell.
 ?I.C.P. = probably isolated clear plaques but background autplaques make it difficult to be sure.
 ? = too many background plaques to tell.

Table 6.15 Phage release from p.t.'s from experiment D

PHAGE SUPERNATANT	TEST RECIPIENT		
	Su297	Su297A12S	Nu18
Øi.Nu18	-	-	T.L.
Ø7.Su297	T.L.	T.L.	T.L.
Ø8.Su297	T.L.	T.L.	T.L.
Ø-buffer	-	-	-
NTRA-1	-	-	T.L.
NTRA-2	T.L.	T.L.	T.L.
NTRA-3	T.L.	T.L.	T.L.
NTRA-4	T.L.	T.L.	T.L.
NTRA-5	-	-	T.L.
NTRA-6	-	-	T.L.
NTRA-7	T.L.	T.L.	T.L.
NTRA-8	T.L.	T.L.	T.L.
NTRA-9	T.L.	T.L.	T.L.
NTRA-10	I.T.P.	I.T.P.	I.T.P.
NTRA-11	T.L.	T.L.	T.L.
NTRA-12	T.L.	T.L.	T.L.
NTRA-13	T.L.	T.L.	T.L.
NTRA-14	T.L.	T.L.	T.L.
NTRA-15	T.L.	T.L.	T.L.
NTRA-16	T.L.	T.L.	T.L.
NTRA-17	T.L.	T.L.	T.L.
NTRA-18	T.L.	T.L.	T.L.
NTRA-19	T.L.	T.L.	T.L.
NTRA-20	T.L.	T.L.	T.L.

T.L. = turbid lysis

I.T.P. = isolated turbid plaques

- = resistant

the Al2 parent they cannot be Su297 prototrophs which may have contaminated the phage lysate, although all phage lysates were sterile in control plates.

The data in Tables 6.14 and 6.15 show that the p.t.'s from experiment D are more heterogeneous than the p.t.'s from experiment C, in terms of their phage release and sensitivities. Three distinct classes of p.t.'s arose and they can be described as follows:-

Class 1, e.g. NTRA 2, $\phi 1^R$; $\phi 7^R$; $\phi 8^R$.

Class 2, e.g. NTRA 3, $\phi 1^R$; $\phi 7^R$; $\phi 8^S$.*

Class 3, e.g. NTRA 5, $\phi 1^S$; $\phi 7^R$; $\phi 8^S$.

* = sensitive but reduced e.o.p.

Of the 20 NTRA clones, 9 appeared to exhibit an autoplaque phenomenon. In contrast to the clones in experiment C these clones had been repurified to isolated colonies only once on minimal agar, then twice on GSYC, before testing. Perhaps the autoplaque clones represent a phage carrier status where there is no autoimmunity in the host to the phage which it carries in a pseudolysogenic or carrier state (see Barksdale and Arden, 1974). Alternatively, they may represent mixed populations of $\phi 7$ lysogens and non-lysogens.

NTRA 2 is the only example of the Class 1 type of mutant. It shows many characteristics of the p.t.'s from experiment C. A phenotypic phage conversion appears to have occurred as with the TRAl-TRA7 series. However in contrast to the TRAl-TRA7 series, NTRA 2 releases a phage which forms plaques on Su297 (Table 6.15). This phage is probably $\phi 7$.

The p.t.'s of Class 2 e.g. NTRA 3 are the predominant class among the 20 tested. They are $\phi 7$ sensitive, i.e. they are not lysogenic for a phage homoimmune with $\phi 7$. They also retained their natural $\phi 1^R$ since they are derived from a natural $\phi 1$ lysogen. Moreover, when tested against $\phi 8$ they showed a reduced sensitivity to it

compared with the Su297 parent. This was decided purely on the criterion of spot tests but the result was very definitive i.e. isolated plaques as opposed to strong turbid lysis. Why this should be is difficult to understand with this limited data. Nevertheless, it does suggest some differences between $\phi 7$ and $\phi 8$. Interestingly, $\phi 7$.Su297 spot tests on the NTRA series tend to yield clear lysis as opposed to strong turbid lysis on the parent strain. In other words this means that $\phi 7$ probably cannot enter into the temperate state as efficiently in the p.t.'s as in the parent. It is as if the integration site on the genome of the host, or site of maintenance, had been lost or changed in the p.t.'s. Once again the p.t.'s appear to release $\phi 7$. Yet, as with NTRA 3, if there are $\phi 7$ lysogens they should exhibit phage conversion.

Testing the p.t. supernatants against a $\phi 7$ lysogen would not be of any value in deciding whether or not these phages released are homo- or hetero- immune with $\phi 7$ because the strains would presumably be subject to phage conversion anyway. Since ϕi probably recombined with ϕx^d (see last chapter) to generate $\phi 7$ then it is not ridiculous to expect that $\phi 7$ may recombine with the resident ϕi prophage in Su297 to yield new heteroimmune phage in the p.t.'s. It is not impossible either, that the progeny phage from such a recombinational event could plate on Su297 or even act as a pseudolysogenic phage type.

The class 3 p.t.'s are typified by NTRA 5 and show the phenotype ϕi^S , $\phi 7^R$ and $\phi 8^S$. How such a type may arise is difficult to imagine. Perhaps one would expect this class of p.t. to be suicidal since, if they became ϕi^S they would, presumably, autolyse due to the resident prophage or due to the ϕi in their immediate environment on the plate. Alternatively, they may be pseudolysogens. Yet none of these show any autoplaque formation, although this effect is known to be variable (Barksdale and Arden, 1974). NTRA 5 and 6 give a turbid lysis response to ϕi and a similar response

to $\phi 8$. For NTRA 19 the response to ϕi is closer to that of NTRA 1. At least in spot tests all 4 show "normal" levels of $\phi 8$ sensitivity. Because they are resistant to $\phi 7$ this must mean that there is a definite molecular difference between $\phi 7$ and $\phi 8$ rather than just a host range variation (see last chapter). It is not known whether or not $\phi 7$ adsorbs to these p.t.'s. If $\phi 7$ does inject its DNA then the block in $\phi 7$ replication in the host could be due to the presence of a $\phi 7$ specific repressor analogous to that of the λ repressor. If this is so, the repressor(s) has no effect on ϕi or $\phi 8$. NTRA 5 and 6 do not release $\phi 7$ but do release ϕi whereas NTRA 19 probably releases both phages.

None of these p.t. supernatants were tested to see if they would recomplement the A12 marker at high frequency.

c) Analysis of putative transductants from experiment E

The results of the analysis of the p.t.'s from experiment E are presented in Tables 6.16 to 6.18. The antibiogram results suggest that the p.t.'s TRA-B and TRA-i are probably contaminants, rather than rhizobia, whereas all of the other TRA p.t.'s react in the expected fashion. Also, TRA-B was capable of growing on NA and so it is not R.trifolii. Although TRA-i did not grow on NA it was relatively resistant to tetracycline and so is very unlikely to be rhizobial in nature. This is because even R68.45⁺ rhizobia do not show as high a level of tet resistance as this TRA-i isolate.

As with the p.t.'s from experiment D, there appears to be a high frequency of autoplague formation (60% of these isolates). Again, the reason for this is obscure. In the spot tests (Table 6.18), TRA-B failed to release a phage which plates on Su297 or Nul8. This reinforces the contention that TRA-B is a contaminant. However, TRA-i yields low levels of phage capable of plating on Su297 and Nul8. Hence TRA-i was probably a mixed culture. The

Table 6.16

Antibiograms of p.t.'s from experiment E

STRAIN	ANTIBIOTIC						
	S(25)	K(30)	N(30)	E(50)	C(50)	PY(100)	Te(50)
TRA-A	0	0	0	0	8	15	8
TRA-B	5	9	4	0	10	28	19
TRA-C	0	0	0	0	8	18	8
TRA-D	0	0	0	0	9	18	18
TRA-E	0	0	0	0	8	17	19
TRA-F	0	0	0	0	9	18	18
TRA-G	0	0	0	0	9	19	18
TRA-H	0	0	0	0	9	17	18
TRA-i	0	7	3	0	9	12	2
TRA-j	0	0	0	0	9	17	18
TRA-K	0	0	0	0	8	18	19
TRA-L	0	0	0	0	9	18	18
TRA-M	0	0	0	0	8	18	18
TRA-N	0	0	0	0	9	19	17
TRA-O	0	0	0	0	9	17	18
TRA-P	0	0	0	0	9	18	18
TRA-Q	0	0	0	0	9	18	19
TRA-R	0	0	0	0	9	18	18
TRA-S	0	0	0	0	9	18	18
TRA-T	0	0	0	0	8	18	19
12Rev-1	0	0	0	0	9	18	18
Su297	2	3	3	0	8	18	18
Su297A12S	0	0	0	0	9	18	18

inhibition zones are measured in mm.

Table 6.17

Phage sensitivities of p.t.'s from experiment E

STRAIN	LYSATE			
	Øi.Nul8	Ø7.Nul8	Ø8.Nul8	H.S.
TRA-A*	-	C.L./T.L.	-	-
TRA-B	-	-	-	-
TRA-C	-	C.L.	I.T.P.	-
TRA-D	-	C.L.	I.T.P.	-
TRA-E*	-	C.L.	-	-
TRA-F	-	C.L.	I.T.P.	-
TRA-G*	-	C.L.	?I.T.P.	-
TRA-H*	-	C.L.	?	-
TRA-i*	-	S.C.L.	?	-
TRA-j*	-	C.L.	-	-
TRA-K*	-	C.L.	?	-
TRA-L*	-	C.L.	?	-
TRA-M	-	C.L.	I.T.P.	-
TRA-N	-	C.L.	C.L.	-
TRA-O*	-	C.L.	-	-
TRA-P*	-	C.L.	-	-
TRA-Q*	-	C.L./T.L.	-	-
TRA-R*	-	C.L.	-	-
TRA-S*	-	C.L.	-	-
TRA-T*	-	C.L.	-	-
12Rev-1	-	-	-	-
Su297	-	C.L./T.L.	C.L./T.L.	-
Su297A12S	-	C.L./T.L.	C.L./T.L.	-
Nul8	T.L.	C.L./T.L.	C.L./T.L.	-

* = autplaques
 - = resistant
 C.L. = clear lysis
 T.L. = turbid lysis

S.C.L. = slight clear lysis
 I.T.P. = isolated turbid plaques
 ?I.T.P. = probably isolated turbid
 plaques but background auto-
 plaques make it difficult to tell.
 ? = autplaques too extensive to tell
 if there are any plaques from spot
 test.

Table 6.18 Phage release from p.t.'s from experiment E

PHAGE / SUPERNATANT	TEST HOST		
	Su297	Su297A12S	Nu18
TRA-A	T.L./C.L.	T.L./C.L.	T.L.
TRA-B	-	-	-
TRA-C	T.L.	T.L.	T.L.
TRA-D	T.L.	T.L.	T.L.
TRA-E	T.L./C.L.	T.L./C.L.	T.L.
TRA-F	T.L.	T.L.	T.L.
TRA-G	T.L./C.L.	T.L.	T.L.
TRA-H	I.T.P.	I.T.P.	T.L.
TRA-I	S.T.L.	S.T.L.	S.T.L.
TRA-J	T.L.	T.L.	T.L.
TRA-K	T.L.	T.L.	T.L.
TRA-L	T.L.	T.L.	T.L.
TRA-M	T.L.	T.L.	T.L.
TRA-N	-	-	T.L.
TRA-O	T.L.	T.L.	T.L.
TRA-P	T.L.	T.L.	T.L.
TRA-Q	T.L.	T.L.	T.L.
TRA-R	T.L.	T.L.	T.L.
TRA-S	T.L.	T.L.	T.L.
TRA-T	T.L.	T.L.	T.L.
12 Rev-1	T.L.	T.L.	T.L.
Øi.Nu18	-	-	T.L.
Ø7.Su297	T.L./C.L.	T.L./C.I.	T.L.
Ø8.Su297	T.L./C.L.	T.L./C.L.	T.L.

T.L. = turbid lysis C.L. = clear lysis - = resistant

S.T.L. = slight turbid lysis I.T.P. = isolated turbid
plaques

data also show that TRA-N did not release a phage capable of plating on Su297, although it probably released ϕ i. From the phage sensitivity patterns (Table 6.17) it can be seen that TRA-N was the only one of the p.t.'s which was ϕ 8^S to the same extent as the parental Su297A12S. This would be the standard response of a classical type Plkc transductant of E.coli K12 where the transductant is non-lysogenic because the only infectious unit it has received is the transducing particle itself which only carries bacterial DNA.

All of the p.t.'s show ϕ 7 sensitivity but the clear response rather than turbid lysis was noted. To a certain extent this assessment was subjective because the turbid response with the parental strains was not the strong turbid response normally associated with ϕ 7.

The response to ϕ 8 was radically different from the uniform pattern of ϕ 7 sensitivity. The majority of p.t.'s were totally resistant to ϕ 8. Those p.t.'s which shared some ϕ 8 sensitivity only showed it with a low e.o.p. compared with the parental reaction. It has previously been demonstrated that ϕ 8 lysogens of Su297 are multiply resistant (see previous chapter). Almost all of the TRA series have released a phage which is likely to be ϕ 8 yet such strains were ϕ 7 sensitive at the same time as being ϕ 8 resistant (to varying degrees). If ϕ 8 did not confer multiple phage resistance then this would be the typical response of a classical ϕ 8 lysogen, in those cases where complete ϕ 8 resistance was observed. It is interesting that the supernatants of each TRA series p.t. had no obvious effect on the homologous strain, yet the supernatants do cause lysis of Su297. With true lysogens the levels of spontaneous phage release are low (10^3 - 10^5 p.f.u./ml) and since a moderate level of ϕ 8 resistance appears to occur in these p.t.'s then one would not expect lysis to occur even in those which show isolated turbid

plaques against the higher titre Ø8 lysate.

The clone 12Rev1 was from a low m.o.i. assay plate and it showed phenotypic multiple phage resistance. Since it released parent-sensitive phage it was probably a Ø8-lysogen. Because 12Rev-1 was isolated as described above it was probably derived from a Ø8 lysogen of a spontaneous revertant. A spontaneous revertant of Su297A12 reacts in the same fashion as the parental Su297, in every respect.

None of the supernatants of this TRA series of p.t.'s was tested for ability to recomplement the Su297A12 mutant.

General Conclusions

All of these results show that the putative transductants, from these experiments, are very heterogeneous with regard to their phenotypes of lysogenic status, phage release and phage resistance. Indeed the discovery of classes of p.t.'s which appear to be lysogenic for either Ø7 or Ø8 but which do not exhibit phage conversion to some extent may contradict claims (Barnet, 1968; Barnet and Vincent, 1970; this study) that lysogenisation with such phage necessarily leads to conversion. It is clear then that a gradation of p.t. phenotypes can arise from Ø7 or Ø8 mediated "transduction"; ranging from "converted", phage-non-releasing prototrophs on one hand to "semi-converted", or non-converted, prototrophs on the other. It is equally clear that a model for the biogenesis of such heterogeneous phenotypes is liable to be necessarily vague at this stage of investigation of this ill-defined system.

Models for the formation of putative transductants

(1) Definition of the problem

Because more than one auxotrophic marker was apparently "transduced" in these experiments it can be said from the outset that any model for the formation of the p.t.'s will have to explain the generalised nature of the "transduction". It is possible to generate generalised transducing phages by either standard infection lysates (e.g. Plkc, Lennox, 1955) or by induction of lysogens (e.g. Plclm c100, Rosner, 1972). However, restricted transducing phages such as λ and Ø80 (Hayes, 1968) generate their transducing particles by aberrant excision from the prophage state and so the formation of transducing particles from these phage is dependent on induction of lysogens. Correspondingly, the restricted transducing phages cannot yield generalised transducing lysates either by induction of lysogens

or by lytic infection. Therefore, the models of transducing phage production as developed for λ , $\phi 80$ (for review see Franklin, 1971 and Hayes, 1968) and the rhizobiophage $\phi 16-3$ (Svab et.al., 1978) cannot be applied convincingly to the $\phi 7/Su297$ system. It seems reasonable therefore to attempt to model the formation of p.t.'s in the $\phi 7/Su297$ system on other systems which exhibit generalised transduction, but which can also form particles which are hybrids of phage and bacterial DNA.

(2) The P1 model and the P22 model

Although generalised transducing phage like P1 (Lennox, 1955) and P22 (for review see Hayes, 1968) generate transducing particles which only carry bacterial DNA rather than phage DNA, several specialised transducing derivatives of P1 and P22 have been isolated which are composed of both phage and bacterial DNA (e.g. see Luria et.al., 1960; Kondo and Mitsuhashi, 1964; Jessop, 1972; Stodolsky, 1973; Rae and Stodolsky, 1974; Rosner, 1975; Mise and Arber, 1976; and Iida and Arber, 1977). In both P1 (Iida and Arber, 1977) and P22 (Tye et.al., 1974) linear DNA is packaged into infectious phage particles by a "headful" packaging mechanism analogous to that proposed for T₄ (Streisinger et.al., 1967). For the generation of plaque forming specialised transducing particles the amount of bacterial DNA packaged must not be too great, otherwise the phage genome will lose too much of its terminal redundancy and consequently will be incapable of efficient circularisation after infection (Rosner, 1975). This is an obligate event because redundancy appears to be required for vegetative replication and lysogenisation. At least in some cases, the formation of such specialised transducing particles of P1 may be dependent on the presence of insertion sequences (Mise, 1976;

Iida and Arber, 1977).

Superficially then, this P1/P22 model provides a basis for the creation of the type of hypothetical vector which is likely to be responsible for the p.t.'s i.e. a hybrid vector which can carry some phage and some bacterial genes but which is not necessarily formed by the induction of lysogens. However, transductants formed from such specialised transducing derivatives of generalised transducing phages generally react as classical heterogenotes (e.g. Luria et.al., 1960), whereas generalised transductants are usually stable (Zinder, 1953). Such a model of specialised transduction would be inadequate therefore to explain partial phage conversion and the ability, or inability, to release phage in the p.t.'s. Hence, alternative models have to be considered.

(3) Pseudolysogeny and the Woods-Thomson model

Several bacterial genera exhibit the phenomenon of pseudolysogeny, or cryptic lysogeny, in which the pseudolysogen is stably lysogenic for a phage which does not express an immunity function (Baess, 1971). These genera include Proteus (Krizsanovich, 1973; Coetzee, 1974), Achromobacter (Thomson and Woods, 1974) and Mycobacterium (Grange, 1975). The phages from such pseudolysogens generally exhibit many features in common with the Ø7-related phages including the ability to induce lysogenic conversion (Coetzee, 1974, 1977; Woods and Thomson, 1975); a biphasic response to thermal inactivation (Thomson and Woods, 1974) and a restricted host range (Woods and Thomson, 1975). Phages from such pseudolysogens can also be capable of generalised and specialised transduction (e.g. Krizsanovich-Williams, 1975; Coetzee and Krizsanovich-Williams, 1976; Woods and Thomson, 1975). Transducing phages which simultaneously convert the recipient cells have also been isolated (Coetzee, 1977). How-

ever there would appear to be quite a remarkable resemblance between the data found for the Ø7 and Ø8 mediated "transductants" and the results from similar work on Achromobacter (Woods and Thomson, 1975).

Woods and Thomson (1975) found that Achromobacter sp.2. was cryptically lysogenic for a phage called α 3a. Lysogens of α 3a became phage converted (Thomson and Woods, 1974) and α 3a acted as a generalised transducing phage (Woods and Thomson, 1975). Moreover, the transductants produced from α 3a were very heterogeneous with respect to their prototrophic stability; phage sensitivity patterns and the host range of their spontaneously released phage. Among the 20 transductants examined by Woods and Thomson, some clones were sensitive to α 3a; some were totally resistant; some were initially resistant but became sensitive or semi-sensitive on subculture, and one class erratically and spontaneously released phage, perhaps in a fashion analogous to the phenomenon of autoplague formation. Hence, phage α 3a was capable of generating a variety of transductants which exhibited a gradation of phage conversion characteristics and various degrees of stability of lysogeny and phage release. This is indeed remarkably similar to the findings with Ø7 and Ø8 in Su297. Woods and Thomson proposed an hypothesis to explain such a range of phage characteristics of the transductants. In this hypothesis the generalised transducing particles contained some bacterial as well as differing amounts of phage DNA. The amount of phage information, varying from particle to particle, could determine to what extent the deficiencies of the defective, cryptic prophage in the recipient cells could be complemented. Hence, among the prototrophic transductants, the variety of phenotypes with respect to phage sensitivity patterns and phage release

could be viewed as a manifestation of the relative degrees of complementation of the resident, defective phage functions.

Generalised transducing phages can package DNA by a headful mechanism from a terminal of randomly fragmented resident genome as well as the phage genome concatenates (Thomas, 1967). It is likely, also, that infection of a cell by a generalised transducing phage leads to fragmentation of the bacterial chromosome, probably due to an endonuclease. It has been proposed (Yamamoto, 1978) that if a random endonucleolytic cut is introduced into a prophage genome by such a process as generalised transducing phage infection, that the host cell recombination function may ligate the superinfecting transducing phage genome, or its segments, with a segment of the prophage at a terminal of the fragmented chromosome. From such a model Yamamoto was capable of explaining the biogenesis of novel recombinants between serologically unrelated generalised transducing phages. Hence, the Yamamoto model could be used to explain the biogenesis of a variety of generalised transducing phages in cryptic lysogens of Achromobacter, which give rise to transductants of such diverse phenotypes (Woods and Thomson, 1975).

It is known that Su297 is lysogenic for at least 2 phages (Barnet, 1968), one of which (ϕ_i) is very closely related to ϕ_7 and ϕ_8 (Barnet, 1968; this study). It may therefore be possible to explain the varied behaviour of the ϕ_7 and ϕ_8 mediated "transductants" of Su297 with a combination of the Yamamoto hypothesis and the Woods-Thomson model. Generalised transduction could occur if the phages (ϕ_7 and ϕ_8) packaged their DNA by a headful mechanism. Assuming ligation of random segments of bacterial DNA to varying segments of phage DNA as envisaged in the Yamamoto model and the Woods-Thomson hypothesis, (Yamamoto, 1978; Woods and Thomson, 1975) then such generalised transducing particles could transduce recipient cells

to prototrophy while simultaneously providing the Su297 cells with varying fragments of the $\phi 7$ or $\phi 8$ genome. Although $\phi 7$ and $\phi 8$ lysogens of Su297 appear to be converted (Barnet, 1968; Barnet and Vincent, 1970; this study) it is possible that the infection of Su297 by incomplete $\phi 7$ or $\phi 8$ genomes may lead to the inability or partial ability to express phage conversion; as found with $\alpha 3a$ mediated transductants of Achromobacter s.p.2. (Woods and Thomson, 1975) and, indeed, in this study. Correspondingly, such transductants could show a variety of phage release phenotypes, varying from no phage release to the release of novel recombinant phages.

The analysis of the $\phi 7$ and $\phi 8$ mediated putative transductants in this study must be necessarily vague at this point in time. It does seem clear, however, that there are analogues of the remarkable behaviour of these "transductants" in other bacterial phage/host systems. Indeed the similarity with the $\alpha 3a$ /Achromobacter s.p.2 system of Woods and Thomson is particularly striking; and a great deal remains to be done to define the molecular biology of such phage/host interactions. Grange, (1975) stated that a more intense study of bacterial pseudolysogeny states might help to explain certain aspects of latency and persistence in mammalian host-virus relationships. This study has shown that, indeed, there are interesting features in common between the $\phi 7/\phi 8$ /Su297 "transduction" system and the generalised transducing system of the pseudolysogen phages of Achromobacter (Woods and Thomson, 1975). This is therefore another example of an interesting, yet ill-defined, area of molecular biology which an intensive study of the Su297/Su297 systems could help to illuminate.

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Post script: a critique of the "technological fix" ideology

In Chapter I it was suggested that the idea of solving the food problems of the third world by a feat of genetic manipulation is an idea that is both naive and dangerous. The reasons why this idea is scientifically naive were discussed in the general introduction. Nevertheless, a variety of commentators still tentatively, or strongly, suggest that, when the technical solution(s) to the scientific problems associated with the engineering of nitrogen fixation are discovered, food production and availability to third world countries will increase (Child, 1976; Evans and Barber, 1977; Burris, 1977; Postgate, 1978). Indeed this idea of a technological solution (technological fix) to the world's food problems permeates the whole of society so comprehensively that it manifests itself in popular journals e.g.

"Cereals that could provide their own
fertilizer (from genetic engineering)
are beyond doubt the biggest prize of
all in the gifts of the new biology -
far bigger in terms of lives to be saved
than even the conquest of cancer or a cow
that could digest sawdust."

(Harpers Magazine, U.S.A.)

Implicit in this belief, or hope, is the idea that the present, and future, "food problem" is one of inadequate food production for an ever increasing global population. However it has been argued (Moore Lappe and Collins, 1977; Manning, 1977; McCutcheon, 1979) that the production of food for the total world population is no problem at all and that most attitudes as to the cause(s) of hunger are based on complete myth (Moore Lappe and Collins, 1977). The cycle of food production and consumption is very complex and is affected by a variety of biological, political and economic factors (Manning, 1977). Moore Lappe and Collins (1977) argue that for all the importance of such abiological and biological factors the major cause of hunger is not the low productivity of the land but the fact

that hunger is inextricably linked to poverty. This is because the poor cannot own land and therefore cannot dictate what crops, if any, are to be grown on such land. Therefore Moore Lappe and Collins view the hunger problem as a reflection of land ownership and they have discussed, and rejected, the technological fix as a solution to such problems.

"But when a new agricultural technology enters a system shot through with power inequalities, it profits only those who already possess some combination of land, money, credit worthiness and political influence."

(Moore Lappe and Collins, 1977)

It can easily be shown that, even without any new "green revolution", malnutrition and starvation need not exist at the present moment. Accepting the technological fix of genetic engineering for nitrogen fixation as the answer, assumes that food productivity and population are the problems. As discussed above, this is not so and therefore the preoccupation that some scientists and popular magazines have with the engineering of nitrogen fixation as a solution to hunger is ridiculously naive.

However the projection of a technological fix to the so called "food problem" is also dangerous. The reason for this is that such ideas become embedded in the psychology of the layman (via the popular press) and, unfortunately, remain in the ideology of science (Enzenburger, 1976; Ciccotti et.al., 1976; Rose and Rose, 1976). The layman is therefore led to an impression that all such problems have a technical or scientific solution, even when the facts can be used to argue that a non-scientific solution is already available! This tends to create an undue dependence on future technological "fixes". This prevailing attitude of a technological fix for the second "green revolution" can therefore be regarded as one aspect of the ideological basis of scientism (see Cameron and Edge, 1979),

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and may divert attention from the root causes of hunger. In summary, then, without political change the genetic engineering of nitrogen fixation will not radically affect global hunger. Therefore it is not genetic engineering that should be the point of focus for this "problem", but politico-economic engineering.